The Triumph of MALDI-TOF Mass Spectrometry and New Developments in Tandem Mass Spectrometry for Clinical Microbiology

Chapter 3:

Applications of MALDI-TOF Mass Spectrometry in Clinical Diagnostic Microbiology.

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Introduction

Laboratory results account for a large part in physicians' management of patients. While the majority of the laboratory results are available the same day of hospital admission, microbiology analysis may be associated with longer time to results. A suspicion of infection is generally based on clinical symptoms and signs, some of them being highly unspecific (1; 2). Indeed, a similar clinical presentation can be caused by different etiologies and different microorganisms. As a consequence empirical treatments made of broad-range antibiotics are often started (3; 4). In bacteriology and mycology, a large part of the diagnostic is culture-based, a sensitive approach (when the sampling is achieved before antibiotics introduction) which is semi-quantitative or quantitative and provides a pure isolate for species identification and antibiotic susceptibility testing (AST), which will allow adapting the antibiotic treatment if needed. The main disadvantage of culture-based approaches is the time to positivity which varies according to the microorganism growth. When a culture is positive, the identification of the incriminated microorganism may rely on 1) colony morphology and microscopic appearance determined by Gram staining or other specific staining, and 2) biochemical identification; some rapid enzymatic and antigenic detection assays allow a rapid identification; however these methods are restricted to a limited number of microorganisms. High throughput automated systems for subculture-based microorganism identification, which represent a large majority of biochemical identifications are still time consuming because of the need of a subculture.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) represents one of the most accurate, reliable and fast method for the identification of bacterial strains from positive cultures and therefore largely replaced all other previously used approaches for microbial identification. It proves to be much cheaper than nucleic-acid based methods such as polymerase-chain reaction plus sequencing, fluorescence in situ hybridization (FISH) or microarrays.

The principle of MALDI-TOF MS is to generate the mass spectrum profile of a sample that consists of a mix of proteins with different masses. Matrix-assisted laser desorption ionization (MALDI), is a method of proteins embedding in a specific matrix that will facilitate the ionization achieved by a laser. The ionized proteins are subsequently separated according to their time of flight (TOF), a function of their mass and charge. A detector will generate a mass spectrum (MS) according to the calculated time of flight. MALDI-TOF MS has been initially applied for the identification of microorganisms directly from whole cells which made it easy to implement in the routine work flow of clinical microbiology laboratory from colony obtain on agar plates. MALDI-TOF MS success largely relies on easy to use instruments with friendly software accessible to non-chemist. MALDI-TOF MS was further applied for

microorganism's identification from other samples, including positive blood cultures and for various applications such as typing and antibiotic resistance determination. In this chapter, we intend to summarize the main applications of MALDI-TOF MS in clinical microbiology.

Principle of microorganisms identification using MALDI-TOF MS

Soft ionization and mass spectrometry applied to microorganisms identification

MALDI-TOF MS identification includes three major steps: 1) the sample (whole cells or protein extract) is deposited on a metal plate and embedded in a matrix that crystallizes the analytes. The sample is then bombarded by brief laser pulses that achieved the ionization by proton transfer from the matrix, which results in positively charged analytes (MALDI), 2) the ions formed by this process are accelerated in an electrostatic field and directed in the flight tube in which they are separated according to their time of flight (TOF), a function of their mass-to-charge ratio (m/z), which is the result of the square of the drift time $m/z=2t^2K/L^2$ (m = mass, z = number of charges on ion, t = drift time, L = drift length, K=kinetic energy of the ion) (Figure 1). To avoid ion collisions in the flight tube, a high vacuum is generated by a pump, before laser pulses; this takes 1-2 minutes and 3) a mass-spectrum (MS) is eventually generated by proteins detection at the exit of the flight tube. The mass spectrum is composed of peaks of specific mass to charge ratio with different intensities, which corresponds to a reproducible fingerprint of a defined microorganism (5). Some characteristic mass peaks are shared by phylogenetically related bacteria and serve for the identification at the genus of species level (6; 7). The identification is achieved by the comparison of the mass-spectrum with a database of reference mass-spectra generated with well-classified bacterial strains.

Biomarker proteins

Mass spectrometry (MS) is a technology initially developed for the analysis of small molecules in chemistry. The use of mass spectrometry for bacterial identification has been first proposed in 1975 due to the fact that different bacterial extracts were associated with unique protein spectra (8). Rapid identification of microorganisms from intact colonies using MS relies on the ability to generate ions from various proteins including ribosomal proteins. Matrix-assisted laser desorption ionization (MALDI), a soft ionization techniques that uses short UV laser pulses (generally around 337 nm), does not degrade extracted proteins. Holland and colleagues reported for the first time that MALDI TOF MS could be applied directly on whole cells to achieve identification by comparison of the MS obtained with either archived reference spectra or with spectra co-generated with cultures of known bacteria (7). Batches of blinded bacterial colonies from agar plate were mixed with the matrix and air-dried, before MALDI-TOF

MS. The obtained spectra were compared to blinded spectra. A number of high-mass ions corresponding to bacterial proteins could be used for species-species matches (7). This method was confirmed by several other studies and used for rapid bacterial identification from whole bacterial cells (6; 9; 10).

Biomarker proteins that constitute the mass spectrum have a molecular mass lower than 15 KDa. A large majority (~50%) of the proteins used for identification are ribosomal proteins due to their abundance and to the fact that the organic solvents and the acidic conditions that are used for cell lysis promote their extraction (11; 12). The results proposed by the identification software correspond to the more closely related species in the database and is associated with a score that integrates the number of concordant peaks as well as the quality of the spectrum. This score enables to accept the identification at the genus or at the species level or to reject it according to threshold established by the manufacturer.

Current commercial MALDI-TOF MS instruments

The success of MS in clinical microbiology relied on the commercialisation of instruments requiring limited hands on time for the sample preparation and overall requiring limited skills for data analysis as they integrated identification software and databases. This allowed the use of MS by clinical microbiologist and not only by chemist or biochemist.

The BioTyper mass spectrometer systems (Bruker Daltonics) are the most largely used. They allow identification from bacteria or fungal cells and provide the possibility to implement the database with spectra generated with the users own isolates. The identification is associated with a log scale of 0 to 3. A score above 2.3 corresponds to a high level of confidence of the identification at species level. A score between 2.0 and 2.3 allows identification at the species level with a good probability. A score between 1.7 and 2.0 suggests a correct identification at the genus level. Score above 1.7 have a limited accuracy.

The Vitek MS (bioMérieux, France) is the former Axima Assurance system (Shimadzu Corporation, Kyoto, Japan). The identification are rated as “ready to report”, “requiring further review” or “no identification made” (13; 14).

The Autoflex II mass spectrometer (Bruker Daltonics) and the Axima Assurance system (Shimadzu Corporation, Kyoto, Japan), which are the most widespread, display similar performances for routine identification of microorganisms both in term of percentage of valid identifications and percentage of correct identifications. A comparative study between the two systems performed on 720 consecutive bacterial colonies obtained in routine clinical laboratory conditions and using the sequencing of the 16S rRNA gene as gold standard, reported 99.1% (674/680) of correct identifications for the Bruker MS system (Bruker) and 99.4% (635/639) for the Shimadzu MS system (15). In a similar study, Martiny et al. reported, 92.7% and 93.2% of correct identification for the Biotyper and the Vitek MS systems,

respectively (*n* = 986) (16). Both systems displayed equivalent performances for yeasts identification; 93.0% (175/188) for the VITEK MS and 92.6% (174/188) for the Bruker Biotyper (17). Other studies, comparing the VITEK MS and the Bruker systems, confirmed the equivalent performances of the two instruments (18-21). The Andromas system (Andromas SAS, Paris, France) uses a software that determine the percentage of similarity of the obtained spectrum with reference strains present in the database which provide a "good identification", an "identification to be confirmed", or an "absence of identification" (22-24). To date only a few number of studies have been published.

Automated colony picking

Manual colony picking is a major limitation of the throughput of MS identification and promote major errors due to inversions which is estimated to occur at a rate of 0.25% (25). The throughput as well as the reduction of major errors should be improved by the emergence of new automated systems for clinical microbiology laboratory such as the Total Laboratory Automation (TLA) system from BD Kiestra (The Netherlands) and the WASP-Lab from Copan Diagnostics' (Italia), including automated colony picking systems (26). Since MALDI-TOF MS is currently used to identify more than 95% of all isolated strains in laboratories were it is implemented, such systems will undoubtedly have a major impact on the overall laboratory technicians workload.

Factors impacting the accuracy of MALDI-TOF MS identifications

The Importance of the database

MALDI-TOF MS accuracy for microorganism's identification largely relies on the representativeness and the quality of the reference spectra database (27; 28). Commercial databases are made of reference spectra expected to cover a maximum of clinically relevant isolates. These spectra are generally made of the cumul of several spectrum obtained for a single isolate or for different isolates. Reference spectra can also be made with isolates from different origins as regards to potential geographic diversity. However a comprehensive database does not exist yet.

Some misidentifications can be due to an insufficient number of reference spectra for a specific species or to the absence of reference spectra. The databases need to be frequently updated with new spectra. Most of the systems allow users to expand the commercial database with spectra generated with "local" isolates. For instance one could implement the database with the spectrum of an isolate unsuccessfully identified using MALDI-TOF MS but that has been undoubtfully identified using other approaches such as 16S rDNA sequencing or other molecular approaches; this has been shown to significantly increase the identification rate (29; 30). To reduce inter-analysis variability, new references spectra should be

generated by the addition of several spectra obtain with a defined isolate. Errors in the database due to a wrong identification of the strain that will serve as reference or taxonomic errors constitute a major source of misidentifications. Thus implementing its own spectra with local isolates requires specific care of the user, especially on the methods used for the identification.

Some BSL3 agents such as *Burkholderia pseudomallei, Brucella suis, Brucella melitensis, Francisella tularensis* or *Bacillus anthracis* are not present in all commercial databases and are included in dedicated databases (31).

Quality of the spectrum and standardization of the pre-analytic

Both for bacteria and fungi, the mass-spectrum is dependent on the age of the colony and the growth medium. Therefore, it is recommended to perform MS identification from fresh colonies. In addition, when implementing the database, it is important that the new reference spectra are made in similar conditions than these used for routine identifications. The quality of the spectrum may also vary according to the investigated species. For instance difficult-to-lyse bacteria such as *Klebsiella pneumoniae*, which is encapsulated, can lead to poor identification score. Other organisms such as *Mycobacteria* spp., *Nocardia spp.*, Actinomycetes, yeasts and fungi need specific extraction protocols (32; 33).

Limit of detection

A sufficient biomass is required to generate a spectrum which quality is sufficient to provide identification. MALDI-TOF MS was initially restricted to colony obtained from culture on solid or liquid media. A major improvement in the diagnostic of bloodstream infections and patient's management is the identification of bacteria and fungi directly from positive blood cultures allowed by the validation of specific protocols aimed to remove erythrocytes and non microbial cells elements and to concentrate bacterial or fungal cells (34). Recent studies also support the potential use of MALDI-TOF MS for microorganism's identification directly from clinical samples such as urines (27).

Errors and misidentifications

Absence of identification can be due to incomplete databases however, a large number of the identification failures result of a poor quality of the mass spectrum obtained for the microorganism of interest that can impair the identification of concordant spectrum in the database. This can be due to a poor biomass or to an inefficient cell lysis or inefficient protein extraction. Erroneous identifications of the reference spectra in the database can be a cause of major errors (25; 28). Misidentifications often come from similar analytes displaying different TOF as a result for instance of an incomplete ionisation. This can be avoided by using a fresh matrix. Finally, the routine identification procedure has to be similar

to the procedure used to obtain the MS for the database as these could impact the quality and quantity of the peaks and impair identification.

Mixed bacterial populations

MALDI-TOF MS has been developed for microorganism's identification from pure culture; identification of a mixed bacterial population can lead to unexpected results. The Gram staining that remains mandatory could disclose a mixed infection. Indeed, identification from mixed populations can generate an inexistent mass spectrum resulting of the sum of 2 or more spectrum from phylogenetically distinct bacteria. Alternatively, the identification software could propose two distinct strains that are not genetically related. Generally the identifications score hardly reach the threshold for high confidence identification at the species level. It is essential that the MALDI-TOF MS identification matches the microorganisms morphotype revealed by the Gram staining and/or suggested by phenotypic characteristics. Thus, identifications obtained from mixed populations should be considered as not definitive; a subculture is recommended for the isolation of the microorganisms and further identification on mono-bacterial colonies.

Closely related species

Some closely related species cannot be differentiated using MALDI-TOF MS even with high quality spectrum using routine procedures (Table 1). This is the case of *Streptococcus* spp. of the "viridians" group. For *Streptococcus pneumoniae* identification, additional phenotypic tests such as the Optochin susceptibility test or bile solubility test are required for definitive identification (35-40). Some strategies, based on the analysis of specific peaks (6949, 9876 and 9975 m/z), allow discrimination between *S. pneumoniae* and group mitis *Streptococcus* species; however, they are hardly applicable in routine (39). The same issue is true for the distinction of *Escherichia coli* and *Shigella* spp. for which even using specific peaks, misidentifications are still occurring (16; 41-43). In the absence of phenotype confirmation such as lactose fermentation or lysine decarboxylase activity the identification of *E. coli* should be presumptive. In practice, one may report the identification of some bacteria as "complex" or "group" of bacteria when the clinical relevance of differentiating some species is questionable and more than one species of these complex are generally obtained with a identification score superior at 2 (Table 1) (44). Nevertheless, such strategies should be updated according to the improvement of the database availability, of new bioinformatic algorithms and of taxonomy evolutions. Based on empirical observations some authors proposed an alternative acceptance criteria for Gram-negative bacteria, which consist in the acceptance of the identification at species level when at least a 0.200 log difference is observed between multiple species present besides a log score ≥ 2.0 (45).

Identification of microorganisms from positive cultures

Identification from positive cultures on solid media

The main application of MALDI-TOF MS in clinical microbiology laboratories is the identification of bacteria from colonies recovered from solid culture media (Figure 2). A standard procedure is suitable for a large majority of the bacteria, which is a convenient for routine identification. Specific identification procedures, that will be discussed later in this chapter and presented in details in dedicated chapter of this book, are needed for some bacteria, such as *Actinomycetes* and *Mycobacteria*.

For routine identification of bacteria, colonies are picked from agar plates using a sterile loop, deposited on the metal MALDI plate and overlaid with a matrix (27; 46; 47). The mixture is air-dried (a step that can be accelerated using a warming plate) and then introduced in the device and submitted to laser pulses. Matrices with different properties can be used: 2,5-dihydroxybenzoic acid (DHB), a-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), ferulic acid (FA), and 2,4-hydroxy-phenyl benzoic acid (27; 46). Each device is generally associated with a specific matrix recommended by the manufacturers. This procedure is straightforward, simple and fast: less than 5 minutes from the colony picking to the final identification. To improve the quality of the spectrum and to increase the accuracy (confidence score) of the identification, the analysis can be achieved following protein extraction using an acetonitrile/formic-acid procedure that promotes the generation of positive ion and increases the number of peaks available for the analysis (25; 48). The formic acid can be added directly on the colonies on the MALDI plate and air-dried before the embedding into the matrix (27). If no identification is obtained, the third option is to achieve a full protein extraction using ethanol and formic acid.

The performance of MALDI-TOF MS identification relies on (i) the number of mass spectra that reach the quality allowing identification and (ii) the number of correct identification. For these two parameters all the commercial devices available display similar performances. The percentage of correct identification ranges from 95% to 99% with the Bruker system (15; 25; 28) whereas Vitek MS system provided 96.2% of correct identifications at the species level (49). It is recommended to avoid working with old colonies and to minimize the number of passages.

Identification from positive blood cultures

Bloodstream infection (BSI) is suspected by physicians on the basis of non specific criteria, which lead to the introduction of empiric antimicrobial treatments. The time to identification of the etiologic agent of a BSI is crucial as the mortality and morbidity of patients is directly dependant on the introduction of the first efficient anti-infectious treatment. Empiric treatments, introduced prior the identification of the

incriminated microorganism and its susceptibility profile, are generally based on broad range antibiotics with a deleterious impact on the profitable microbiota.

Identification from positive blood cultures via a (short) subculture

Positive blood cultures are not suitable for direct identification using MALDI-TOF MS as the bacterial concentration is generally too low (10^6 to 10^9 cells/ml) and because of the presence of an excess of non bacterial proteins that would impair MS identification (50). A first strategy is to perform a subculture of the positive BC in order to obtain a pure culture on agar-based medium, on which the standard MALDI-TOF MS identification methods described above can be applied. However this is a time consuming methods. Indeed, considering that MALDI-TOF MS identification turnaround time (TAT) is lower than 5 minutes, it is regrettable that the time to result since the positive BC is detected is delayed of several hours because of a long subculture. To accelerate the time to result from the detection of the positive BC, an alternative strategy is to achieve a short subculture on agar plate. The efficiency of this procedure is however dependent on the bacterial strain growth rate (27). This approach is particularly suitable for Gram-negative bacteria for which sub-cultures of ≤4, ≤6, ≤8 and ≤12 h yielded 95.2%, 97.6%, 97.6%, 97.6% of correct identifications; in contrast the same incubation time yielded to only 18.6%, 64.0%, 96.5%, 98.8% of correct identifications for Gram-positive bacteria (51). With an incubation period of 5 hours, Verroken et al. reported 81.1 % (727/896) for monomicrobial cultures with misidentifications mainly occurring for yeasts and anaerobes (52). Subculture is especially suitable for laboratories with a large volume of positive BC and with limited technicians as so far subculture independent methods that will be presented below require a lot of hands-on time (Table 2) (34).

Directly from the positive blood-culture vial

In order to reduce the turnaround time (TAT) to final identification from positive BC, several subculture-independent strategies, which principle is to remove non-microbial material (erythrocytes, cells debris) and to concentrate the microorganisms, have been developed and reported in numerous publications. A first method consists in bacterial enrichment from the positive blood culture by centrifugation and erythrocyte lysis with an ammonium chloride solution. This procedure gives a successful identification for 78.7% of the bacterial pellet tested with 99% of correct identifications (53; 54). A method using collection tubes with separator gels in which 1.5 mL from the positive BC are injected before bacterial protein extraction and MALDI-TOF MS analysis gives more than 90% of correct identification (35). A similar method using a tube containing a separator gel and a clot activator leads to 95.3% of correct identification (36). Equivalent results can be obtained with commercial pellet preparation kit (37; 55-57).

The pellet enriched in bacterial cells generated from positive BC for MALDI-TOF MS identification can also be used for antibiotic susceptibility tests (AST) using disc-diffusion methods, E-test (bioMérieux,

France) or automated systems. Prod'hom et al reported 99% and 74% of correct identification for *Enterobacteriaceae* and for staphylococci respectively and less than 1% of errors for AST using the VITEK 2 automated system (58). More recently, another group obtained similar results using saponin and chloride ammonium. Such a strategy has been transposed to the BD Phoenix automated system with 97.9% of correct AST (59). Using a commercial Lysis-Filtration method of bacterial enrichment from positive BC associated with the VITEK MS system, Machen et al. reported a TAT of 11.4 hours until AST result when conventional methods TAT was 56.3 hours (60).

Identification of microorganisms directly from samples

Urines

Ferreira et al., first reported the possibility to diagnose urinary tract infections by direct detection of bacteria in urinary samples (61). The procedure consisted of an initial centrifugation of 4 ml of urine at low-speed (2,000 g) to remove leukocytes, followed by a high speed centrifugation (15,500 g) to obtain a bacterial pellet suitable for MALDI-TOF MS analysis. The performance of MALDI-TOF MS were compared to the performance of culture for the detection and identification of 260 positive urine samples using the automated screening device UF-1000i (bioMérieux, France) based on flow cytometry as reference. Among the 260 samples, 235 corresponded to mono-bacterial cultures with more than > 10^5 CFU/ml. MALDI-TOF MS correctly identified 91.8% (202/235) microorganisms at the species level and 92.7% (204/235) at the genus level (61). In another study, March et al. also reported a threshold of 10^5 CFU/ml for successful identification directly from urine (62). In contrast, Kohling et al. reported that the identification of bacteria directly from urine samples is possible for concentrations as low as 10^3 CFU/ml. The authors also noticed that the presence of human defensin that inserts into the membrane of bacteria and cannot be removed by washing had a negative impact on MALDI-TOF MS identification (63). A screening of positive urines using the automated system UF-1000i followed by direct analysis using MALDI-TOF MS, has been reported to give 94.8% (1381/1456) of correct identifications (64). Altogether, these studies demonstrate the potential of MALDI-TOF MS to detect pathogens directly from urine. However, given the workload associated with such procedures and the number of urines to investigate, this approach is not widely used. Moreover, the impact of such approach on patients management still need to be investigated and might be limited if MALDI-TOF MS rapid identification is not associated with rapid antibiotic susceptibility testing.

Cerebrospinal fluid

To date, no study has addressed the reliability and usefulness of MALDI-OF MS for the detection of pathogens directly from cerebrospinal fluid (CSF). Nevertheless a case report discussed the feasibility of such strategy. In this report, a CSF from an unconscious febrile patient who presented with a nuchal rigidity, was sent to the clinical microbiology laboratory and analysed by MALDI-TOF MS using the same protocol that was described above for urines: low speed centrifugation (2000 rpm for 30 seconds) to remove leucocytes and then high speed centrifugation (13,000 rpm for 5 min) to obtained a bacterial pellet deposited onto the MALDI-plate for analysis. This approach proposed *Streptococcus pneumoniae* as the aetiological agent (65). There is a need of additional studies to determine the reliability and the added value of such approaches.

Microorganisms requiring a specific processing for MALDI-TOF MS identification

While most of the bacteria can be identified from positive cultures using MALDI-TOF MS, some of them would require a specific processing mainly aimed to promote cell lyses. In addition, some microorganisms require an inactivation step for bio-safety reasons. Several chapter of this book are dedicated to the identification of specific bacteria using MALDI-TOF MS, nevertheless in this paragraph, we selected and underlined some of these aspects.

Nocardia and Actinomycetes

Nocardia spp. and Actinomycetes have a cell wall difficult-to-lyse requiring a specific processing prior MALDI-TOF MS identification. Verroken et al., developed a method for identification of *Nocardia* spp. that consists in 30 min of boiling, followed by a full protein extraction using ethanol and formic acid (66). Using this procedure together with an extended homemade database made of 110 isolates, the authors could accurately identify 88% (38/43) of the *Nocardia* strains including 34/43 correct identifications at species level (66). In contrast, only 44% (19/41) of correct identifications, including 10 correct identifications at species level, were obtained using the MALDI BioTyper commercial database (66).

Mycobacteria

The identification of *Mycobacterium tuberculosis* from positive culture is generally achieved by antigen detection and by PCR. Similarly, the identification of nontuberculosis mycobacteria (NTM) can also be achieved by PCR or other nucleic acid-based methods such as DNA-DNA hybridization. Mycobacteria identification using MALDI-TOF MS has been proposed to decrease turn-around time, labor, and cost compared to nucleic-acid based methods. Several procedures including bacteria inactivation and cell

disruption have been developed for the different MALDI-TOF MS systems commercially available with similar performances (67). However, the concordance of *Mycobacterium* species identification using MALDI-TOF MS with molecular-based methods varies according to the studies and the protocols. This is due to the fact that some closely related strains can hardly be distinguished using this method (68). However MALDI-TOF MS may present an interest for laboratories devoid of a molecular diagnosis platform for nucleic acid extraction, amplification, and sequencing or for nucleic-acids hybridization.

Prior MS identification, *Mycobacterium* species need specific sample processing to inactivate the microbes and to achieve cell disruption. Inactivation can be achieved by incubating the bacteria at 95°C for 30 min in 70% ethanol. A sonication step is necessary to optimise cell disruption before protein extraction. Using this procedure on cells collected from 7H11 agar medium, Machen et *al*. reported 82.2% of correct identification at species level and 88.8% at genus level (69). Kodana et *al*., proposed a slightly different procedures in which the heat inactivation is achieved in water with a subsequent ethanol treatment. Alternatively, El Khechine et al. proposed a heat inactivation performed in the presence of Tween-20 followed by a mechanic cell lysis and a full protein extraction; using this method, the authors reported 100% of agreement with conventional molecular methods for 124 clinical isolates including, 87 *M. tuberculosis* strains and 37 NTM strains (70). Cell inactivation and disruption can also be achieved simultaneously by vigorous vortex of the cells during 15 min in 70% ethanol in the presence of glass bead which gave 88.8% of correct identification (69). A heat-independent inactivation/extraction method has been proposed by Lotz et al. in which a cell pellet obtained from positive MGIT or Lowenstein-Jensen culture was inactivated in 70% ethanol, which was sufficient to work outside the biosafety level 3 laboratory (71). The cell suspension was deposited on the MS plate and air-dried before the addition of the matrix (sinapinic acid, 20 mg/ml; acetonitrile, 30%; trifluoroaceticacid, 10%). After the addition of 10 mM ammonium phosphate on the crystallized mixture, the sample was analysed by MS giving 97% of correct identification from Lowenstein-Jensen and 77% of correct identification from MGIT (71). Very recently, another group demonstrated that a 5 min 70% ethanol inactivation may be sufficient for mycobacterial identification (33).

In conclusion, MALDI-TOF MS can provide a same day result from positive mycobacteria cultures. Inactivation of the cells should be ensured in order to perform the analysis outside BSL3 laboratories. The inactivation procedure is generally not sufficient for cell disruption, which requires a specific step. However, misidentifications can occur for closely related strains or for rare species for which there are not enough reference mass-spectra in the database (68; 71). This is the case for *M. abscessus*, *M. massiliense*, and *M. bolletii* and for *M. tuberculosis*, *M. bovis*, *M. bovis BCG*, *M. microti*, and *M. africanum*. In conclusion, *Mycobacteria* spp. can accurately be identified using MALDI-TOF MS.

Yeast and Fungi

Yeast identification using MALDI-TOF MS can be achieved directly from whole cells using a procedure similar to the standard bacterial identification procedure from agar plates. The two main commercial MALDI-TOF MS systems display similar performances for the identification of yeast at species level with up to 89.8% for the Bruker Biotyper MS system and 84.3% for the VitekMS system (17; 72). These performances are comparable to the VITEK 2 system (17). A full extraction method that consists in a pre-treatment in 70% ethanol and formic acid/acetonitrile protein extraction gave 99% of correct identification using the Bruker Daltonics MALDI BioTyper software (73). The age of the colony is expected to impact the accuracy yeast identification; however Goyer et *al*., reported similar results for colonies grown, 48h or 72h, with respectively 95.1% and 96.6% of correct identification from chromogenic agar media (74). Nevertheless, caution is warranted for much older colonies ($\geq$ 5 days).

The identification of invasive filamentous fungi using protein MALDI-TOF MS is more complex than for bacteria and yeast, because of their complex life cycle. Indeed, different life stages, spores, conidiophores and mycelium, can be present on the same colony. A first strategy is to mix the different growth stages present in a single colony, and then to perform protein extraction for MALDI-TOF MS analysis (22). Specific sample preparation methods are also required to achieve cell lysis and to improve protein extraction. In contrast to yeast identification, the accuracy of MALDI-TOF MS for the identification of filamentous fungi is largely dependent on the growth medium, the age of the colony and the growth stage. Repeated identification can be necessary. Using a simplified extraction method and an extended database generated with 55 species of *Aspergillus* spp, *Fusarium* spp and *Mucorales* spp, De Carolis et *al*. reported 96.8% (91/94) of correct identification (75). Samples were prepared by placing 1µl of a suspension of mycelium and conidia onto the MALDI-plate from which direct protein extraction was achieved using ethanol/alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid (75).

Dermatophytes identification at species level is generally not necessary because antibiotic susceptibility profile is generally shared by all the members of a defined group. If identification at species level is required, an *in situ* identification by microscopy and PCR is possible. The identification of dermatophytes using MALDI-TOF MS is impaired by the fact that microorganisms are difficult to recover after culture. Dermatophytes require a long (4 to 5 days) culture, with a significant impact on protein expression, which can impact the MALDI-TOF MS analysis. Nevertheless some protocols have been proposed. As for other filamentous fungi, it is recommended to pool the different growth stages present in the same colony or to multiply the identifications within the same colony. Using an extended database, De Respinis et al. reported 60 to 100% of correct identification of Dermatophytes depending on the species (76). More

recently, Wang et al., also successfully used MALDI-TOF MS, not only to identify yeasts at species level, but also dermatophytes (77).

Detection of antimicrobial resistance

Carbapenemase detection

Carbapenemases are enzyme produced by some *Enterobacteriaceae* and some non-fermenting Gram negative bacteria that can hydrolyse carbapenems, a class of broad spectrum antibiotics. The first approach for the identification of carbapenemases producing strains using MALDI-TOF MS is based on the detection of ertapenem hydrolysis corresponding to a shift or a disappearance of the antibiotic specific peak on the mass spectrum. Kempf et *al*. focused on *Acinetobacter baumannii*, a bacterium that mainly produces 3 families of carbapenemases OXA-23-like, OXA-24-like and OXA-58-like. On the mass spectrum obtained by MALDI-TOF MS, carbapenemases production resulted in the reduction of a 300.0 m/z peak corresponding to native imipenem and the increase of a 254.0 m/z peak corresponding to the natural metabolite of imipenem in the supernatant of bacteria incubated with imipenem during 4 h (78). This method displayed 100% of sensitivity and 100% of specificity for the detection of carbapenemase producing strains. Carvalhaes et *al*. monitored ertapenem degradation through the disappearance of the 475 and 497 m/z peaks corresponding to the native form and to the monosodium salt form of the molecule. This strategy identified 72.4% (21/29) of the carbapenemase-producing strains including 100% of the KPC-2 and 100% of the SPM-1 (100%) but hardly OXA-23-producing *Acinetobacter baumannii* strains (79). A similar method described by Vogne et al., monitoring ertapenem degradation, showed 100% of sensitivity and 100% of specificity for the detection of carbapenemase producing strains among *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter cloacae*, *Providencia stuartii*, *Serratia marcescens*, *Enterobacter aerogenes*, *Hafnia alvei*, *Klebsiella oxytoca*, *Proteus vulgaris*, *Morganella morganii*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and *Aeromonas* species (80). The simple approach proposed by Vogne et *al*. exhibited a short turnaround time (~60 minutes) as it used commercially available antibiotic disc, which reduced the hands on time. In addition this approach could benefit from the high stability of the antibiotic molecule in such commercial formulation.

Methicillin-resistant *S. aureus*

Approaches, more related to typing, aims to identify genotype associated with specific resistance profiles using MALDI-TOF MS. They have been mainly used for the distinction between methicillin-susceptibility *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) (81; 82). These approaches correspond to the detection of biomarker proteins associated with resistance strains. However, Edwards-

Jones et al., suggested that the identification of some MALDI-TOF MS characteristic peaks could be used for the distinction between methicillin susceptibility and methicillin resistant *S. aureus* (83).

Vancomycin Resistant Enterococci

By comparing spectra obtained from vancomycin sensitive *Enterococcus faecium* and vancomycin resistant isolates (VRE) Griffin et al., demonstrated that a 5,945 Da peak could distinguish sensitive and resistant strains. Another peak at 6,603 Da could distinguish vanA- and vanB-positive isolates (84). This likely represents strains-type identification rather than antibiotic susceptibility markers and should be taken with caution.

Other aspects of the antibiotic resistance detection based on strains typing will be addressed in the next chapter. Altogether these studies suggest that MALDI-TOF MS is suitable for antibiotic resistance determination. However these studies need to be extended to more bacterial isolates and need to be reproduced in other location to determine if the accuracy of these approaches is impacted by the local epidemiology of resistance isolates.

Detection of bacterial virulence factors

Because of its accuracy, MALDI-TOF MS has the potential to detect specific peaks corresponding to virulence factors such as toxins. Gagnaire et *al*., demonstrated that MALDI-TOF MS applied to *S. aureus* whole cells could detect two peak at 3005 Da and 3035 Da corresponding to the *S. aureus* delta-toxin, a biomarker of the activity of the *agr* (accessory gene regulator) system (85). In *S. aureus*, the *agr* system regulates the expression of numerous virulence factors and pathogenesis-associated determinant, which represents a therapeutic target (86; 87).

MALDI-TOF MS has also been proposed for *S. aureus* strains typing or for the detection biomarkers of the most virulent toxigenic isolates. The Panton-Valentine leukocidin (PVL), a toxin produced by some strains of MSSA and MRSA is associated with recurrent skin and soft tissue infections as well as necrotizing pneumonia. The gene encoding the PVL brought by phages has a high potential to spread among isolates. Bittar et *al*. initially reported the identification of two peaks of 4448 and 5302 Da associated with PVL positive isolates (88). However, a second study revealed that they were no reliable association between the 4448 and 5302 Da peaks and the presence of PVL; PVL positive strains were identified by chance because of similar mass profile due to their high relatedness (89).

Konrad et *al*. reported the correct identification of 99.1% (116/117) of toxigenic *Corynebacterium* sp. (*C. diphtheriae, C. ulcerans* and *C. pseudotuberculosis*) among non toxigenic species using the MALDI-TOF

MS system Microflex LT mass spectrometer (Bruker Daltonics) and the BioTyper 2.0 identification software. This result was not obtained directly from colonies but after rapid protein extraction using ethanol and formic acid. The discordant result was a *C. tuberculostearicum* isolate that could not be identified at species level neither using the MALDI-TOF MS (log (score) of 1.8), or by sequencing of the *rpoB* gene or using the API Coryne gallery (90). When the score was above 2.0 the negative predictive value and the positive predictive value were both 100% attesting the high reliability for the detection of potential toxigenic *Corynebacterium* species.

Typing and Clustering

At present, the reference methods for isolates typing are nucleic acid based methods such as multi-locus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) because of their excellent discriminatory power that only whole genome sequencing can surpass. However, these methods can be time consuming and expensive which makes more rapid and cost-effective methods needed.

MRSA typing

S. aureus typing by MLST or PFGE generally relies on the *spa* gene (*S. aureus* protein A gene). Wolters et *al*., reported that the analysis of 13 characteristic peaks allow the distinction of the major *S. aureus* MRSA lineages by MALDI-TOF MS with a discriminatory indices of 0.770 (95% CI 0.671–0.869) comparable to the discriminatory indices of genotyping using the *spa* gene 0.818 (95% CI 0.721–0.915) (82). Lu et *al*., proposed an approach based on peak allowing the distinction between *S. aureus* SCC*mec* types IV and V isolates that are mainly community-associated (CA) MRSA and SCC*mec* types I–III isolates that are hospital-associated (HA) MRSA (81). Josten et *al*. analysed the mass spectrum profile of 401 MRSA and MSSA strains and identified peaks that differentiate the main *S. aureus* MRSA and MSSA clonal complexes that could be used for the distinction of sensitive and resistant strains (91). Altogether these studies suggest that MALDI-TOF MS is a reliable method for the typing

Enterobacteriaceae typing

Noteworthy typing using MALDI-TOF MS requires a precise calibration of the instrument using highly conserved peaks and generally requires a database of references spectra representing the major lineages of the species of interest. However, Christner et al. developed an approach that includes the identification of biomarkers peaks for MALDI-TOF MS typing during an outbreak. This approach identified two peaks, one at m/z 6711 and one at m/z 10883, that correctly classified 292/293 isolates of Shiga-toxin producing *E. coli* during the 2011's outbreak that occurred in northern Germany (92). The reliability of the MALDI-TOF MS for typing isolates directly from whole cells has also been demonstrated for *Yersinia*

enterocolitica (93) and *Salmonella enterica* (94). Interestingly, strain typing using MALFI-TOF MS generally relies on the analysis of selected specific biomarker only.

Mycobacteria typing

By comparing the mass spectra of *M. abscessus*, *M. massiliense* and *M. bolletii* isolates, Suzuki et *al*., identified a cluster of *M. massiliense* strains closely related to *M. abscessus* due to the similarity of their mass spectra indicating that MALDI-TOF MS could also be used for mycobacteria strain typing and clustering (95).

Application of MALDI-TOF Mass Spectrometry in Clinical Virology

Clinical virology aim at: i) viruses detection, ii) identification (including typing and epidemiology) and iii) resistance detection. For these purposes, the clinical virology laboratories rely on cell culture, electron microscopy, serology and PCR with satisfy results. Nevertheless, several groups have addressed the possibility to use the MALDI-TOF MS in these different aspects of clinical virology, and when possible, they address the added value (96).

The first approach is the detection of biomarker viral proteins from infected cell cultures. Using this strategy, Calderaro et al., reported the detection of *Picornaviridae* from infected cell culture using the viral protein VP4 as biomarker (97)

La Scola et al., demonstrated that giant viruses (capsid sizes of 150 to 600 nm) could be characterized, using the MALDI-TOF MS analysis of viral particles. The results obtained correlated with results provided by the sequencing of the *polB* gene (98). However, this method requires specific equipment and is time consuming (cell lysis, filtration, concentration by centrifugation, rinsing, ultracentrifugation), which impairs its use in routine.

Another approach applied for influenza viruses detection consisted in the capture of viral particles using magnetic nanoparticles functionalized with H5N2 specific antibodies with subsequent MALDI-TOF MS analysis of the complexes nanoparticles/viruses to detection of the hemmaglutannin protein HA (99). This allowed H5N2 detection with no cross reactivity with H5N1 viruses.

PCR-Mass assay

MS technologies have been applied to the analysis of nucleic acid post amplification. This approach that combine PCR and MALDI-TOF MS, also known as PCR-Mass assay, allows the simultaneous detection of several amplicons at the same. In addition, this technology is sensitive enough to detect a single nucleotide polymorphisms or a nucleic acid modification such as methylation.

Application of PCR-Mass assay in clinical bacteriology

The Sequenom MassARRAY platform (Sequenom) is an instrument dedicated for nucleic acid analysis using the PCR-Mass assay approach (100). The amplification is achieved in the presence of deoxyuridine triphosphate (dUTP) instead of deoxythymidine triphosphate (dTTP). The amplicons are then fragmented with uracil-DNA-glycosylase and the obtained fragments are analyzed by MALDI-TOF MS (101). The mass pattern is then compared with a database or with a reference spectrum. Bacterial identification can be achieved by using PCR targeting the 16S rDNA (101). This technique is also reliable for microbes typing or to detect mutations at the level of a single nucleotide that are associated with antibiotic resistance (102). The sensitivity also allows detecting methylation variations (103).

This technology can be applied to the detection uncultivable microorganism directly from clinical samples as demonstrated for *Bordetella* species detection by targeting the 16S rRNA gene (101).

Application of PCR-Mass assay in clinical virology

PCR-Mass assay is an extremely sensitive and specific approach that has been applied to clinical virology for the detection, identification and quantification of viruses as well as for the genotyping and the detection of drug resistance (96). Noteworthy, this technology allows simultaneous analysis of several amplicons. Using two multiplex PCRs followed by MALDI-TOF MS analysis of the amplicon, Sjoholm et *al*., could detect and identify any herpesviruses directly from various body fluid including bronchoalveolar lavage, conjunctival fluid, sore secretion, blister material, plasma, serum, and urines with an average concordance of 95.6% (86.4-97.2%) with PCR results. (104). Piao et al. reported the simultaneous detection and identification of 8 enteric viruses (enterovirus 71, coxsackievirus A16, reovirus, poliovirus, hepatitis E virus, norovirus, astrovirus, and hepatitis E virus) with a detection limit of 100-1000 copies per reaction (105). Discrepant results during the validation of these methods have been associated with false negative results due RNA degradation (105).

The Mass assay technology was also demonstrated to be useful for the high throughput diagnosis of HPV infectious with simultaneous genotyping to differentiate between the high-risk and low-risk types (106; 107). Mass assay has been successfully used for the detection of CMV resistance to ganciclovir in patient

609 treated with this drug by detecting a single point mutation of the viral phosphotransferase (UL97) or the
610 viral polymerase UL54 associated with, ganciclovir resistance (108).

611 PCR-ESI MS

612 PCR electrospray ionisation mass spectrometry (PCR-ESI MS) is a technology that associates DNA
613 amplification by PCR and consecutive analysis of the obtained amplicon by MS (109; 110). Basically,
614 nucleic acids extracted from clinical samples are amplified using multiple broad-range PCRs. Then, the
615 precise molecular mass of the amplicon(s) is/are determined by ESI/MS. This mass integrated to the
616 amplicon length and to the DNA complementary rule provide the exact base composition (%A, %C, %G
617 and %T) of the amplicons. Pathogens identification is then achieved by comparison with a database (110;
618 111). This technology allows the detection of a broad range and a high number of pathogens: more than
619 750 bacteria and fungi, more than 200 fungi and more than 130 viruses. The bacterial panel include
620 primers for the detection of the resistance genes *mecA*, bla_{KPC}, *vanA* and *vanB*. Compared to current
621 methods of clinical microbiology, PCR-ESI MS have a short turnaround time (~8h) because: i) it can be
622 applied directly on clinical samples without culture due to its high sensitivity and ii) the analysis of the
623 amplicon is achieved by ESI-MS rather than Sanger sequencing. PCR-ESI MS has been tested on various
624 clinical samples such as CSF (112-114) and respiratory tract samples (115-117) with an increased
625 sensitivity when compared to culture. The last platform that has been developed, allow diagnosis of
626 bloodstream infections as it can accept up to 5ml of sample and because of the optimisation of the nucleic
627 acid extraction and the amplification step (118). This device known as the PLEX-ID (Abbott Molecular,
628 Des Plaines, IL) has recently been commercialized under the name Iridica. The Iridica system can detect
629 more than 800 hundred pathogens directly from blood with a sensitivity of 50-91% and a specificity of
630 98-99% (50). PCR-ESI/MS can detect poly-microbial infections and provide informations on the
631 abundance of each micro-organism based on a semi-quantitative analysis. The broad spectrum of this
632 technology together with its extremely low detection limit makes it highly sensitive to contamination.
633 Interventional studies will determine the exact clinical impact of this new technology; especially the
634 impact on antibiotic stewardship.

635 Impact of MALDI-TOF MS in clinical microbiology and infectious disease

636 Time to result

637 A challenge for clinical microbiology is to accelerate the diagnostic when an infection is suspected,
638 especially when most of the other laboratory results are obtained within the first hours of hospitalization

(119). Despite recent developments especially in the domain of molecular diagnostic, culture-based methods remain predominant. Thus, MALDI-TOF MS dramatically reduces the time-lapse between the positive culture and the final identification when compared to subculture-based phenotypic identifications. In a large prospective study, Seng et al., estimated that MALDI-TOF MS identification took approximately 6 to 8.5 minutes for when the time to result was 5 to 8 hours for the Vitek system (BioMérieux, France) and 5 to 20h for the Phoenix system (BD Diagnostic) that rely on phenotypic methods (28). Tan et *al*. also reported a reduction of the time to result for a set of 20 bacteria, with a mean time gain of 1.45 days (120). In particular, the reduction monitored by the authors was ~1.35 days for *S. aureus* and *Enterobacteriaceae* identification. The maximum gain was observed for Gram positive rods, with a reduction of 4.13 days.

Impact on patient management

When a bloodstream infection is occurring, delaying the introduction of an efficient anti-infectious treatment directly impacts patient survival. Despite the emergence of culture independent methods, blood cultures remain the gold standard to determine the etiologic agent of a bloodstream infection (34; 50). A prospective observational study performed in a tertiary hospital that evaluated the impact of MALDI-TOF MS on BC positive with Gram-negative bacteria using a rapid bacterial pellet method reported that the empirical therapy was often inappropriate or too broad. In 35.1% of cases, the rapid identification led to a modification of the empirical therapy (121). In this study, a correct identification at the genus level was obtained in 86.7% (143/165) of mono-microbial infection (121). In their study, Martiny et al. demonstrated that MALDI-TOF MS was helpful to confirm suspected contamination in 37.50% of cases for paediatric patients (122).

Impact on rare pathogenic bacteria and organisms difficult to identify

Rare microorganisms can be defined as microorganisms with less than 10 reports designating them as human pathogens on the Pubmed database (123). Alternatively, some organisms were difficult to identify accurately because of the phenotypic proximity with other organisms (124). The identification of these pathogens is generally time- and labor-consuming as often requires gene sequencing. MALDI-TOF MS allows us to reported the first case of bacteremia caused by *Comamonas kerstersii*, a nonfermenting microorganisms that was difficult to distinguish from other *Comamonas* species or closely-related *Delftia* spp. in the pre-MALDI era (125). For both rare microorganisms and difficult-to identify microorganisms MALDI-TOF MS significantly reduces the use of DNA sequencing as we reported in a study where we obtained nearly 50% of accurate identification for this type of organisms (126).

The accuracy of MALDI-TOF MS identification might reveal the real incidence and the pathogenic role of some rare/difficult to identify microorganisms (20; 123; 124).

Anaerobes

The diagnosis of anaerobic bacteria is generally long because of their slow growth and its accuracy is often limited due to their limited biochemical activity. In addition anaerobes are often occurring in polymicrobial infection resulting in multiple different species in the same sample. In a study conducted between 2010 and 2011 on 283 anaerobic bacteria, Nagy et *al*. reported 77 % of correct identification at species level and 10.95% of correct identification at genus level using the MALDI-TOF MS (127). Moreover, among 544 anaerobic bacteria isolated from clinical samples, La Scola et al. reported 61% of successful identification at species level using the MALDI-TOF MS compared to 39% of identification by 16S rRNA gene sequencing (128). This demonstrated the potential of the MALDI-TOF to reduce the number of time consuming and expensive 16S rRNA gene sequencing for anaerobes identification as reported for rare microorganisms' identification. Noteworthy, the rate of successful identification of anaerobes using the MALDI-TOF MS, meaning the percentage of identification that obtain a sufficient confidence score is dependent on the representativeness of the database (129-132).

Identification of protozoan parasites

While waiting for confirmatory studies, several publications suggest that MALDI-TOF MS may be used for the identification of protozoa. The analysis of mass spectra obtained with intact *Giardia lamblia* and *Giardia muris* cysts identified common peaks as well as species specific peaks that could be used for MALDI-TOF MS identification of these two microorganisms (136). Cassagne et al., demonstrated that *Leishmania* promastigotes could successfully be identify at the species level, from in vitro culture using a homemade reference mass spectra database made of the main *Leishmania* species known to cause infection in human. This strategy correctly identified correctly identified 66 of the 69 *Leishmania* promastigotes isolates tested with log score values superior to 2. Two isolates failed to generate interpretable mass spectra while one isolate identified a *Leishmania braziliensis* isolate as the closely related *Leishmania peruviana* isolate (137). To evaluate the ability of MALDI-TOF MS to determine the subtype of enteric *Blastocystis*, Martiny et al., constructed a database of specific protein signatures of five *Blastocystis* subtypes. This approach gave correct subtype determination for 19 axenic cultures of various *Blastocystis* subtypes used to challenge the database (138). By analyzing the MALDI-TOF MS spectra of *Entamoeba histolytica* and *Entamoeba dispar*, Calderaro et al., identified five peaks that could be used to discriminate the two species (139).

Identification of ticks and fleas

Yssouf et al., demonstrated that mass spectra obtain from leg extracted hemolymph was reliable for the identification of ticks at species level. The proof of concept was achieved using a homemade database made of 5 *Rickettsia*-free tick species (*Rhipicephalus sanguineus*, *Hyalomma marginatum rufipe*, *Ixodes ricinus*, *Dermacentor marginatus* and *Dermacentor reticulatus*), 1 infected tick species (*Amblyomma variegatum* infected by *Rickettsia africae*), and other arthropods, including mosquitoes, lice, triatomines and fleas (140). Using a second database enriched in hemolymph from *Rickettsia africae*-infected ticks the authors could reliably distinguish non-infected and infected specimens (140-142). The same group also demonstrated that fleas could be identified based on MS generated from various body part (143).

Costs

So far, the price and maintenance expenses of MALDI-TOF MS instruments are high. Nevertheless MALDI-TOF MS was shown to be inexpensive in comparison with phenotypic and genotypic methods of identification. Indeed, the cost for the identification of a given isolate can be less than 1.5 euros per identification using MALDI-TOF MS versus 5.9-8.23 euros for the Vitek System (28; 133). A prospective costs analysis study compared MALDI-TOF MS protocol with standard identification protocol. For this study the supplementary tests necessary for the identification of some bacteria such as *Streptococcus pneumoniae* and *Shigella*, the additional cost due to repeated MALDI-TOF MS analyse, the instrument maintenance expenses and microorganism prevalence were including (120). By integrating all these data, the authors anticipated a 56% of reduction of reagent and labour costs for one year. In another retrospective cost assessment study after MALDI-TOF MS implementation, an overall 89.3% cost saving was obtained (134). This group mentioned also an important decrease in waste disposal and a reduction in subculture medium expense. As we also reported, MALDI-TOF MS has the potential to reduce the expense due to DNA sequencing for some difficult-to-identify isolates (126). In another prospective economical study, we reported that microbial identification was 2.43 fold less expensive with MALDI-TOF MS than standard methods. However, this ratio varied from 0.70 to 7.0 according to the bacterial species. Some identifications (for example, urinary *Escherichia coli* identified on chromogenic culture media and confirmed by a simple spot indole test) were cheaper with standard identification than by MALDI-TOF MS. The cost savings will thus depend on the epidemiology and the prevalence of each species encountered in the clinical laboratory and the laboratory habits and procedures (Heiniger et al. unpublished data). Using a mathematical model, it was calculated that implementation of MALDI-TOF equipment and identification would be cost effective if more than 5'300 to 8'000 strains were identified yearly. With more than 20’000 identifications/year, Tran et al. estimated that the initial cost of the

instrument would be neutralizer after about three years (120). Martiny et al. have shown that the implementation of the MALDI-TOF MS technology is an opportunity for the mutualisation of processes such as analytical platforms with important cost savings (135). Further cost savings may occur in a near future with the automation of the preparation of MALDI-TOF target plates.

Conclusions

MALDI-TOF MS is one of the major revolutions that occurred in clinical microbiology laboratory in the last decades, nearly rendering obsolete all biochemical identification galleries, since more than 95% of all bacterial identification are nowadays performed using MALDI-TOF MS. At the exception of some body fluids such as urines, MALDI-TOF MS is still dependant on positive culture (agar plate or blood-culture for instance) because more than 10^4 cells (approximately 10^7 cells/mL) are required to generate a good quality spectrum suitable for identification (144). Further development such as microorganism enrichment by affinity or increasing the sensitivity limit of MALDI-TOF MS might make it reliable for identification directly from samples. In the meantime, new approaches such as PCR-MALDI-TOF MS and PCR-ESI MS will extend mass spectrometry applications in clinical diagnostic microbiology as these nucleic-amplification based methods are suitable for the diagnosis directly from clinical samples including blood (50). MALDI-TOF MS also proved its reliability for other applications such as antibiotic resistance detection and typing; however most of the procedures developed so far are still difficult to achieve in routine and are restricted to specialized laboratories. Because MALDI-TOF MS is reliable at determining bacterial biomass, one could expect that in the future this could represents a reliable read-out for rapid antibiotic susceptibility testing based on the impact of an antibiotic on bacterial growth. As it is an innovation with increased accuracy and undisputed convenience impacting the identification of rare microorganisms and allowing the discrimination between closely related organisms, MALDI-TOF MS might reveal the real incidence and the pathogenic role of some organisms.

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Tables and Figures

Table 1: Example of interpreted results as currently reported in our laboratory

Complex of bacteria	Species
Acinetobacter baumanii complex	*A. baumanii* *A. calcoaceticus* *A. nosocomialis* *A. pittii*
Bacteroides fragilis complex	*B. caccae* *B. eggerthii* *B. fragilis* *B. ovatus* *B. stercoris* *B. thetaiotaomicron* *B. uniformis* *B. vulgatus*
Burkholderia cepacia complex (according to clinical situation, identification to species level may be clinically relevant)	*B. ambifaria* *B. anthina* *B. arboris* *B. contaminans* *B. cenocepacia* *B. diffusa* *B. dolosa* *B. lata* *B. latens* *B. metallica* *B. multivorans* *B. pyrrocinia* *B. seminalis* *B. stabilis* *B. vietnamiensis* *B. ubonensis*
Citrobacter freundii complex	*C .brakii* *C. freundii*

	C. gillenii *C. murliniae* *C. rodentium* *C. sedlakii* *C. serkmanii* *C. youngae*
Enterobacter cloacae complex	*E. asburiae* *E. cancerogenus* *E. cloacae* *E. dissolvens* *E. hormaechei* *E. kobei* *E. ludwigii* *E. nimipressuralis*
Haemophilus influenzae complex	*H. haemolyticus* *H. influenzae*
Klebsiella oxytoca complex	*K. oxytoca* *Raoultella ornithinolytica* *Raoultella planticola* *Raoultella terrigena*
Proteus vulgaris complex	*P. hauseri* *P. penneri* *P. vulgaris*
Streptococcus bovis complex	*S. equinus* *S. gallolyticus* *S. infantarius* *S. lutetiensis* *S. pasteurianus*
Streptoccus mitis complex	*S. gordonii* *S. infantis* *S. massiliensis* *S. mitis* *S. oralis*

	S. peroris *S. parasanguinis* *S. sanguinis* *S. tigurinus*
Streptococcus salivarius complex	*S. salivarius* *S. vestibularis* *S. thermophilus*

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1300 **Table 2: MALDI-TOF MS applications in clinical microbiology and references**

Application	Comments	Selected references
Standard identification from colonies	Fast, accurate; misidentification associated with absence or incorrect identification of the reference spectra in the database.	(25; 27; 28; 49)
Identification from positive blood culture via a short subculture	Suitable for Gram negative bacteria; not suitable for Gram positive bacteria, anaerobes and yeast.	(51; 52)
Identification from positive blood culture via bacterial pellet	Positive impact on patients managements; misidentification or absence of identification is generally associated with insufficient biomass or mixed bacterial population. High hands-on time.	(34-37; 144-146)
Identification from urines	For culture positive with more than 10^3-10^5 CFU/ml depending on the study; high workload, unknown impact on patients managements.	(61-63; 147)
Identification from cerebrospinal fluid	Proof of concept provided for a meningitidis case	(65)
Alpha-hemolytic streptococci	Misidentification of group mitis streptococci and *S. pneumoniae*	(39; 148)
Beta-hemolytic streptococci	Fastest and more accurate than conventional methods. Errors can occur with rare species	(133)
Aerobic Gram positive bacilli	Accuracy depend on the database	(23; 90)
Shigella spp./E. coli	Routine distinction methods with 100% of sensitivity and specificity are not available	(16; 41-43)
Anaerobes	Important added value due to the long incubation time required for these microorganisms.	(127-132)
Mycobacteria	Rapid for the identification of NTM. No added value for MTBC complex identification. Need specific inactivation and cell lysis procedure, no added value for laboratory with a molecular diagnosis platform.	(69-71; 149)
Yeast	Accurate, same procedure than bacteria	(17; 24; 150-154)
Fungi	Requires specific extraction procedure to achieve cell lysis. Impacted by the age of the colony and the growth stage.	(22; 75)
Dermatophytes	Variable sensitivity according to the species, limited clinical impact of identification at the species level	(76)
Protozoan parasites	Amoeba *Leishmania* species *Giardia* species	(139) (137) (136)
Ticks and fleas	Proof of concept from hemolymph (ticks) and various body parts (fleas)	(140; 143; 155; 156)
Typing	Can be accurate but still time consuming	(81; 82; 91-93)
Resistance	Essentially for carbapenemase-producing strain identification	(78-80)
Toxin	Requires the identification of the exact virulence factor-associated peak. Misidentification can be due to type associated peaks rather than toxin associated peaks	(85; 88; 89)
Viruses	Detection, identification (including simultaneous detection), typing, nucleic-	(96; 97; 106-108; 157)

	acid methylation	
PCR-ESI MS	Universal (>800 pathogen), detection of mixed bacterial populations, semi quantitative. Low throughput (8 samples/8h), expensive, no interventional studies	(50; 111; 118; 158)

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1303 **Table 3: Added value of MALDI-TOF MS in clinical microbiology**

	Comment	Selected references
Reduced time to results	Gain of 24h and more over conventional methods	(15; 28)
Reduced cost	Cost effective due to reduced cost of identification of a given isolate	(15; 120; 134)
Improved therapeutic management of sepsis	Adaptation of the antibiotic treatment in ~35% of bacteremia involving Gram negative bacteria	(34; 121)
Increasing accuracy	Less than 2% of errors at species level	(25; 27; 28; 49)
Improved identification of specific pathogenic agents	Reduced need for 16S rRNA gene sequencing Discovery of new pathogens	(20; 123; 124; 126)

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Figure 1: Principle of MALDI-TOF MS identification of microorganisms

The sample is first deposited on a metal plate and embedded in the matrix that crystallizes the analytes and bombarded by brief laser pulses that achieved the ionization (MALDI), by proton transfer from the matrix, which results in positively charged analytes. Then the desorbed ions are accelerated by an electrostatic field and directed in the flight tube in which they are separated according to their time of flight (TOF) in the flight tube in which a high vacuum is generated by a pump. Ions are detected at the exit of the flight tube and a software generates a mass-spectrum. The identification is achieved by the comparison of the mass-spectrum with a database of reference mass-spectra (adapted from Croxatto et al., FEMS Microbiol Rev. 2012 Mar;36(2):380-407).

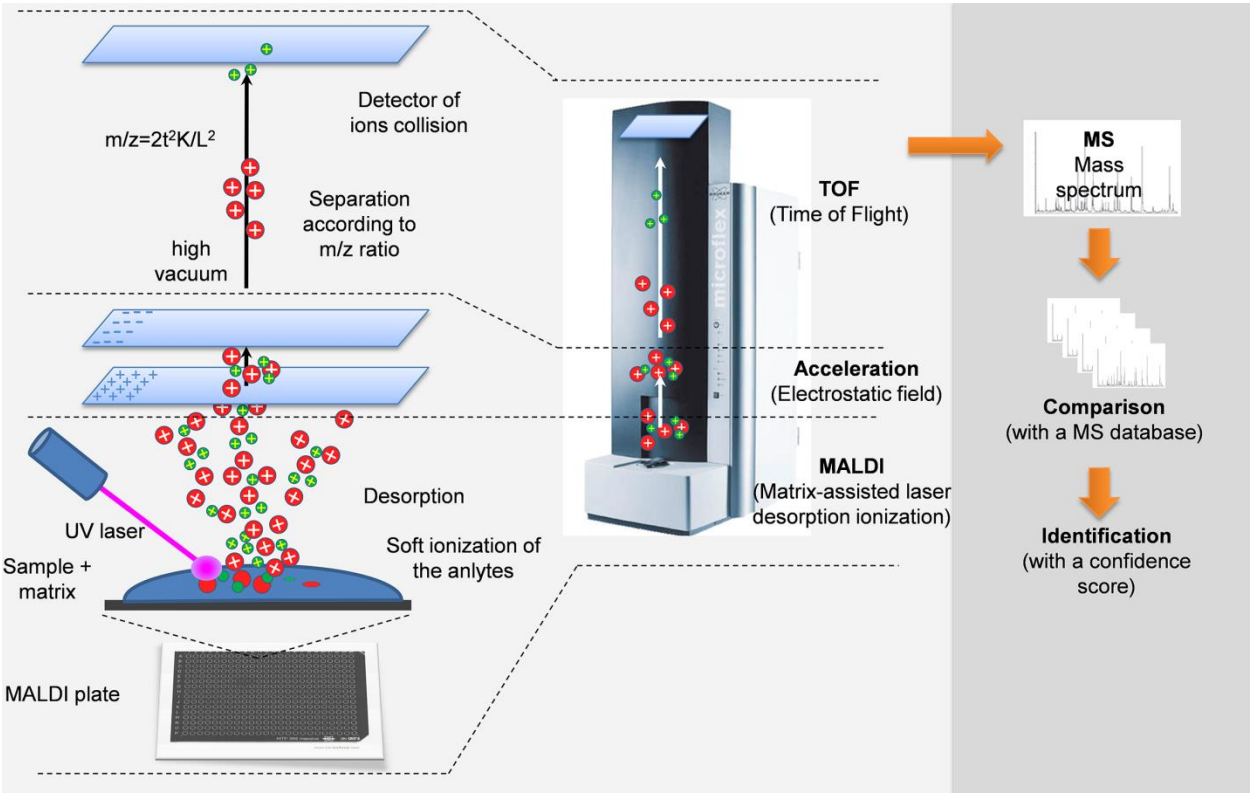

Figure 2: Application of MALDI-TOF MS in clinical microbiology