종설
Lab Med Online
Vol. 5, No. 4: 176-187, October 2015
http://dx.doi.org/10.3343/lmo.2015.5.4.176

임상미생물학 LMO

MALDI-TOF MS의 임상검사실 내 적용과 미생물검사의 패러다임 변화

MALDI-TOF MS: Its Application in the Clinical Laboratory and a Paradigm Shift in Clinical Microbiology

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In the past decade, clinical microbiology underwent revolutionary changes in methods used to identify microorganisms, a transition from slow and traditional microbial identification algorithms to rapid molecular methods and mass spectrometry (MS). Earlier, MS was clinically used as a highly complex method that was adapted for protein-centered analysis of samples in chemistry laboratories. Recently, a paradigm-shift happened when matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS was implemented to be used in microbiology laboratories for rapid and robust methods for accurate microbial identification. Two instrument platforms, marketed by well-established manufacturers, are beginning to displace automated phenotypic identification instruments and in some cases even genetic sequence-based identification practices. This review summarizes the current role of MALDI-TOF MS in clinical research, in diagnostic clinical microbiology laboratories, and serves as an introduction to MALDI-TOF MS, highlighting research associated with sample preparation, algorithms, interpretations, and limitations. Currently available MALDI-TOF MS instruments as well as software platforms that support the use of MALDI-TOF with direct specimens have been discussed in this review. Finally, clinical laboratories are consistently striving to extend the potential of these new methods, often in partnership with developmental scientists, resulting in novel technologies, such as MALDI-TOF MS, which could shape and define the diagnostic landscape for years to come.

INTRODUCTION

Mass spectrometry (MS) is an analytic technique used to analyze the mass-to-charge ratio of various compounds [1]. Different methods, based on various ionization and detection systems, have been developed. The most widely used method to date for the analysis of biomolecules is matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS). It is based on the ionization of cocrystallized sample material by short laser pulses. The ions are accelerated and their time of flight is measured in a vacuum flight tube. MALDI-TOF MS has been successfully used in research to determine the mass of proteins and peptides, in addition to identifying previously unknown proteins. MALDI-TOF MS has contributed to the diagnosis of tumors, rheumatoid arthritis, Alzheimer's disease, and allergies, through the identification of specific biochemical markers. The first attempts to identify microorganisms using MS were performed as early as 1975 [2]. However, these experiments suffered from irreproducible results due to variability caused by culture conditions and media. Only with the discovery of MALDI-TOF MS in the 1980s did the analysis of relatively large biomolecules, including large ribosomal proteins,

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Received: March 16, 2015
Revision received: May 13, 2015
Accepted: May 14, 2015

This article is available from http://www.labmedonline.org

become possible [1]. The latter are less influenced by culture conditions, allowing MALDI-TOF MS to be consistently used to differentiate bacterial species [3]. In recent years, MALDI-TOF MS has been implemented in routine laboratories, and utilized as a completely new approach for the identification of bacteria and yeast.

MALDI-TOF MS IN CLINICAL MICROBILOLOGY

1. Protocol, Algorithm, and Interpretation

Since MALDI-TOF MS is a very sensitive technique, only a small amount of microbial biomass is required for analysis (for bacteria, 10^4-10^6 CFU). To identify a microorganism, the sample is mixed with 1 μL of matrix solution and placed on the steel surface of the target plate to dry. The matrix solution (cinnamic acid or a benzoic acid derivate) cocrystallizes with the sample on the target plate. A typical target plate can hold between 16 and 384 samples. The loaded target plate is inserted into the machine, where it is then transported to the measuring chamber. Within the mass spectrometer, a high vacuum has to be continuously maintained. However, upon insertion of the loaded target plate, air is introduced into the system and the vacuum must be reestablished before sample analysis can be performed. Once a sufficient vacuum has been created, the individual samples are exposed to short laser pulses. The laser's energy vaporizes the microorganism together with the matrix, leading to ionization of the (ribosomal) proteins. An electromagnetic field, created by a potential of about 20 kV, accelerates the ions before they enter the flight tube. The time of flight (TOF) of the analytes to reach the detector at the end of the flight tube is precisely measured. The degree of ionization as well as the mass of the proteins determines their individual TOF. Based on this TOF information, a characteristic spectrum is recorded and constitutes a sample fingerprint, which is unique for a given species.

For species-level identification, the size range generally used is between 2 and 20 kDa as samples within this range were found to be very stable and with a strong signal-to-noise ratio. Interestingly, this size range is dominated by ribosomal proteins, which ionize well, provide accurate spectra, and are only minimally influenced by microbial growth conditions. The computer software automatically compares the collected spectra with a reference databank containing a wide variety of medically relevant isolates. The measured spectra are subject to method-inherent noise and therefore, will never be identical for individual isolates. The software that compares the spectra generates a numerical value (score value) based on the similarities between the observed and stored data sets. This score value provides information about the validity of the identification. A score value above 2.0 is generally considered to be a valid species-level identification. Values between 1.7 and 2.0 represent reliable genus-level identification. Furthermore, the software displays additional results next to the best match for plausibility checks. Current algorithms allow the entire computational analysis to be performed in near real time [4, 5]. Therefore, if only one sample is to be measured, it can be processed in 5-7 min and provides a species-level identification. If a target plate containing 96 isolates is used, results can be obtained in about 1 hr starting from the time-point at which the first sample is loaded on the plate. After completion of the analysis process in the MALDI-TOF MS, the used target plate is removed from the machine. Disposable target plates can be discarded in the regular laboratory waste, while reusable versions are cleansed for further use. Most recommended cleaning procedures start by treating the plate with ethanol and trichloroacetic acid solutions, and include mechanical cleansing steps. Generally, a quick cleaning protocol, using 70% ethanol for 5 min and subsequent mechanical cleaning with detergent and a cloth, is sufficient for regular workup and produces significantly less chemical waste than other protocols. Further, more aggressive cleaning protocols can be performed on a weekly basis.

Matrices used in MALDI-TOF MS experiments are generally crystalline solids with low vapor pressure that can easily become volatilized to form ions in a vacuum (as in the context of MALDI-TOF MS). The chemical matrix is mixed in excess with a clinical sample and allows for production of intact, gas-phase ions from large, nonvolatile, and thermally labile compounds, such as proteins. The matrix plays a key role by absorbing the laser light energy and causing a small part of the target substrate to vaporize. Matrices should possess certain characteristics, such as strong absorbance at laser wavelengths used to facilitate ionization, stability in a vacuum (to force an interaction with the simultaneously ionized clinical specimen), an ability to ionize the clinical specimen, solubility in solvents that are compatible with the clinical specimen (to create an effective matrix-specimen mixture), and a complete lack of any chemical reactivity with the clinical specimen (to avoid unwanted alterations or damage to peptides con-

tained within the sample.) In the case of MALDI-TOF MS, which uses a UV laser, the matrix molecule must also have a strong chromophore as part of its composition to help absorb energy, thus preserving the protein fragmentation. Chromophores are selected based on their ability to absorb specific laser wavelengths, resulting in electronic excitation of the matrix.

2. Applications for Intact Cells (with Culture)

Early studies evaluating the use of MALDI-TOF MS for microbial identification focused on the ability of the technology to accurately determine the identity of whole microorganisms isolated from agar-based culture. MALDI-TOF MS eliminated the need for protein extraction methods prior to analysis, allowing intact microorganisms to be simply spotted onto a solid plate, mixed or overlaid with a matrix compound, and cocrystallized, which facilitates the dissociation and ionization of bacterial proteins [2, 6]. The intact cell (IC) method provided a new and simple mechanism for rapid analysis of bacterial components based on the generation of specific spectrums, and facilitated accurate microbial identification and characterization [2, 6, 7]

Due to the simple procedure for sample preparation, IC MS became an attractive alternative to phenotypic and genetic methods of microorganism identification. Several studies supported the observation that IC MALDI-TOF MS was sufficiently sensitive to differentiate closely related microorganisms [8], and perhaps even discriminate between different strains of the same microorganism or phenotypically similar microorganisms [9-11], providing new avenues for genus-, species-, and strain-level identification.

However, as analysis of microorganisms by MALDI-TOF MS became more commonplace, it became apparent that the IC method was not always appropriate for all specimen types, in spite of its relative simplicity; problems with spectral generation from some microbes [12, 13] and biosafety issues [14, 15] arose. In an effort to improve spectral generation and be compliant with biosafety regulations, modified versions of sample preparation methods have been reported for different groups of microorganisms, and range from on-plate inactivation using formic acid and matrix to full-scale protein extraction using ethanol-based methods. Two platforms from well-established commercial manufacturers are available for MALDI-TOF MS identification of bacteria and yeast, and names of hardware and software are shown in Table 1. Spectral databases are often marketed as part of a proprietary system, as opposed to a publicly accessible open platform, and are constructed and maintained by their respective manufacturers. A majority of these databases can be expanded to accommodate spectral entries that are not included in marketed versions. The ability to add spectra and construct custom databases is important for further discriminatory analysis using MALDI-TOF MS, including strain typing and epidemiological investigations, and algorithms and interpretation forms are shown in Fig. 1. As each proprietary system uses its own algorithms, databases, software, and interpretive criteria for microbial identification, numerical data (i.e., spectral scores) from different commercial systems are not directly comparable [16]. Therefore, comparative analysis of MS systems are usually performed, using the final identifications in each system's interpretive algorithms. MS platforms from different manufacturers are summarized in Table 2 (according to the US FDA 510(k) summary of October 28, 2013).

3. Applications without Culture

Due to its exquisite sensitivity, MALDI-TOF MS provides an attractive means, either in place of—or in concert with—PCR-based strategies, for direct detection of pathogens from clinical material, as no amplification of the target material is required. Also, the ability of both molecular and proteomic approaches to identify targets in clinical samples can be enhanced by preliminary processing of the samples, removing some of the elements that can inhibit analysis. A few reported methods show promise for the identification of microorganisms using MALDI-TOF MS without subculture [17-20]. Although recent evaluations showed promise, the methods were limited by the need for a large number of cells; adaptations of the MS methods showed successful identification

Table 1. MALDI-TOF MS: Algorithm and interpretation

	Bruker Daltonics System	bioMérieux System
Instruments (Manufacturer)	Microflex (Bruker Daltonics)	Axima Assurance (Shimadzu)
Software (Manufacturer)	BioTyper (Bruker Daltonics)	IVD: Vitek MS (bioMérieux) RUO: Saramis (bioMérieux, originally by Anagnos-Tec)

http://dx.doi.org/10.3343/lmo.2015.5.4.176

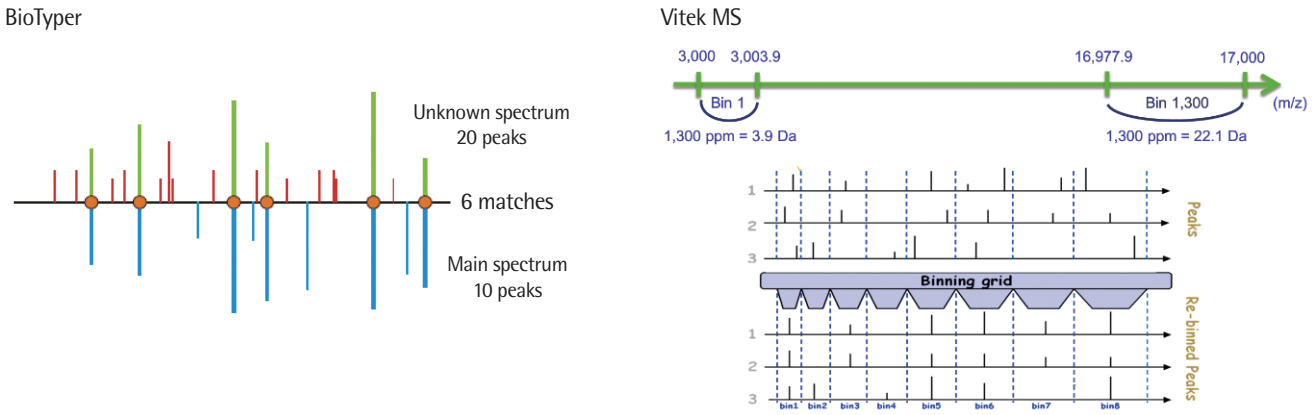

Fig. 1. Algorithms and result sheets of two commercially available MALDI-TOF analysis platforms.

Table 2. Summary of two commercial MALDI-TOF analysis systems (based on US FDA 510(k) summary of October 28, 2013)

	BioTyper	Vitek MS
	Similarities	
Sample type	Columbia blood agar with 5% sheep blood	Columbia blood agar with 5% sheep blood
	Trypticase soy agar with 5% sheep blood	Trypticase soy agar with 5% sheep blood
	Chocolate agar	Chocolate polyvitex agar
	MacConkey agar	Campylosel agar
		MacConkey agar
		Modified Sabouraud Dextrose agar
		ChromID CPS
Type of test	Automated Mass Spectrometry System	Automated Mass Spectrometry System
Matrix method of testing	α-Cyano-4-hydroxycinnamic acid	α-Cyano-4-hydroxycinnamic acid
	Bacteria: Direct testing If after initial analysis the log(score) is reported at < 2.00, organisms are processed using the extraction procedure.	
Result Reporting	Organism identification is reported with high confidence if the log(score) is ≥ 2.00.	A single identification is displayed, with a confidence value from 60.0 to 99.9, when one significant organism or organism group is retained.
	An organism identification is reported with low confidence if the log(score) is between 1.70 and < 2.00.	"Low-discrimination" identifications are displayed when more than one but not more than four significant organisms or organism groups are retained
		When more than four organisms or organism groups are found, or when no match is found, the organism is considered unidentified
Recorded mass range	2,000-20,000 m/z	2,000-20,000 m/z
	Differences	
Culture Age	Bacteria growth should be between 18 to 36 hr	Bacteria and yeast growth should be between 24 to 72 hr
Calibration	Bruker US IVD Bacterial Test Standard (BTS)	E. coli ATCC 8739
MADI Target Plate	US IVD 48 Spot Target	VITEK MS-DS Target Slides
	• 48 positions reusable steel targets	• 48 positions disposable plastic targets
MALDI-TOF MS instruments	Bruker microflex (benchtop)	Shimadzu AXIMA® Assurance MS (floor standing)

for only approximately 80% of blood cultures [21]. In general, repeated preparation of samples with scores of < 1.7 followed by measurement with MALDI-TOF MS did not improve the identification. Interestingly, in a subgroup of blood-culture preparations with a lower score (< 2.0), there was a strong correlation with elevated numbers of leukocytes in the original blood samples [22].

1) Urine

Urine is a good candidate for direct detection of pathogens from clinical material, as it does not contain normal flora and

there are almost no host proteins in the sample. At the same time, urinary tract infections (UTIs) are normally monomicrobial and characterized by high concentrations of pathogens during the course of infection [23, 24]. Prior to the use of MALDI-TOF MS for the diagnosis of UTIs, the presence of blood and bacteria in the urine was reported to interfere with urinary proteomic analysis, specifically altering key peptide-mass signals in the sample [24, 25]. An additional report similarly suggested that bacterial overgrowth of the urine could hamper proteomic analysis, and recommended that samples be immediately centrifuged and stored at 4℃, and have boric acid or sodium azide added to prevent bacteria from overgrowing [26]. Which specimen handling conditions are necessary for optimal identification of bacteria in urinary samples remains to be seen, as a high bacterial burden within urine samples could potentially simplify the detection of the bacteria at the expense of the rest of the urinary proteome. To prepare the urine samples for analysis, low-speed centrifugation was utilized to remove leukocytes, followed by high-speed centrifugation to collect bacteria in the sample, and these intact cells were analyzed by MALDI-TOF MS. In a large study using this protocol, it was possible to correctly identify pathogens at the species level, directly from urine samples, at a rate of 91.8% (significant bacterial load, $>10^5$ CFU/mL) [23]. In the study's analysis of 269 samples designated as positive by urine particle analysis (urine microscopy), 20 were positive in a screening device, but negative by both culture and MALDI-TOF MS analysis (using the Bruker Biotyper 2.0 database). A second study by the same group further addressed the issue of sample preparation with respect to direct urine samples prior to MALDI-TOF MS analysis. An additional study by Köhling et al. [27] investigated the ability of MALDI-TOF MS to identify bacteria directly from urine specimens, and compared the accuracy of this method to that of either phenotypic or molecular methods of identification. Although using a smaller sample set (n=107) than that used in other studies discussed in this section, the authors similarly determined that MALDI-TOF MS was a reliable methodology for direct detection of bacteria from urinary specimens. It is possible that the reliability of direct detection by MALDI-TOF MS may be applicable to samples with bacterial densities as low as 10^3 CFU/mL. The most recent studies regarding the direct analysis of urinary specimens were aimed at incorporating MALDI-TOF MS into the laboratory workflow in conjunction with the urinalysis section of the clinical laboratory. In the report of a comprehensive study by Wang et al. [28], flow cytometry was utilized as a prescreening method to eliminate negative urine samples. Samples determined to be positive for the presence of bacteria ($>10^5$ CFU/mL) by flow cytometry were processed for bacterial identification by MALDI-TOF MS. Samples were differentially centrifuged to remove leukocytes from the suspension, followed by high-speed centrifugation to pellet bacteria. The bacterial pellet isolated from the aqueous urine was subsequently treated with formic acid and acetonitrile, and extracted proteins were analyzed by MALDI-TOF MS (using the Bruker Biotyper 2.0 database) for bacterial identification. The resulting identifications were compared to identifications derived from cultured bacteria by using phenotypic methods, with discrepant identifications being resolved by 16S rRNA gene sequencing. Of the 1,456 samples from patients with UTI symptoms included in the study, 932 (64%) were determined to be negative for the presence of bacteria. An informative result (no bacteria present or correct bacterial identification) was obtained for 1,381 of the 1,456 cases (94.8%). Among the 430 positive samples, the results for eight were found to be discrepant between the analyses by MALDI-TOF MS and the VITEK-2 system (bioMérieux), and all eight identifications by MALDI-TOF MS were confirmed by 16S rRNA gene sequencing. It seems that MALDI-TOF MS has the potential to be used with great success for the direct identification of bacteria in urine samples. However, while tremendous work has been dedicated to the direct analysis of urine specimens by MALDI-TOF MS, there are a number of questions that remain to be answered. Both Ferreira et al. [23] and Wang et al. [28] reported that MALDI-TOF MS could not accurately identify mixed bacteria present in urinary specimens. It remains to be determined if improvements to the MALDI-TOF MS databases will allow the accurate identification of mixed bacteria in urinary specimens. Additionally, no standardized methodology is currently available for the processing of specimens prior to analysis by MALDI-TOF MS.

Simple protein extraction (i.e., using formic acid or ethanol-acetonitrile) has been demonstrated to be cost-effective and fast, significantly enhances the ability to correctly identify bacteria from urine specimens by MALDI-TOF MS, and would be a reasonable step to include in a standardization of processing methods prior to MALDI-TOF MS analysis. Moreover, many clinical laboratories are already performing flow cytometry on urine, with urine cultures often being submitted for reflex testing following a positive

http://dx.doi.org/10.3343/lmo.2015.5.4.176

microscopic observation. There is a possibility that MALDI-TOF MS could be added as an option for reflex testing and performed following a positive urine microscopic test. Irrespective of future in the specimen-processing workflow, MALDI-TOF MS currently represents a robust and accurate technology for the identification and characterization of single bacterial species present in direct urine specimens. Thus, MALDI-TOF MS presents itself as a true alternative to more lengthy and expensive culture-based microbial identification systems, especially in the outpatient setting where resistance testing is not required.

2) Cerebrospinal Fluid

Similarly to urine, cerebrospinal fluid (CSF) has been used in proteomic profiling for the diagnosis of disease. The presence or absence of specific proteins in a patient's CSF was used as a biomarker for a number of neurological disorders. Bacterial meningitis represents one of the most serious and clinically significant manifestations of bacterial infection, and a disease where fast and accurate detection of the causative bacterial agent is paramount. Currently, the detection of bacterial pathogens responsible for meningitis is accomplished via Gram staining of the CSF to detect the presence of bacteria. Samples generating Gram-negative smears are cultured to rule out the presence of circulating bacteria, while patients whose smears show positive Gram staining are immediately treated with broad-spectrum antibiotics based upon the Gram stain result, until a definitive identification can be reached and targeted antimicrobial therapy administered. There are very few reports in the literature describing the use of MALDI-TOF MS for the direct identification of bacteria from CSF. Following sample acquisition, the CSF was processed in a manner similar to that discussed in the preceding section for urine samples: low-speed centrifugation to remove leukocytes followed by high-speed centrifugation to pellet bacteria, solubilizing of the pelleted debris in formic acid-acetonitrile, centrifugation, and analysis by MALDI-TOF MS using the Biotyper 2.0 database (Bruker). According to Nyvang Hartmeyer et al. [29], the identification generated by MALDI-TOF MS analysis was interpreted as being valid species-level identification following manual manipulation of the data, although the automatic analysis would have allowed for only a statistically confident genus-level identification. Irrespective of the genus or species level of identification, the use of this technique in combination with Gram staining and traditional bacterial culture could represent an important turning point in the diagnosis of bacterial meningitis, increasing sensitivity and decreasing time to diagnosis, and allowing for targeted and aggressive antibiotic therapy for a patient population that is critically ill. This is an application of MALDI-TOF MS that warrants significant further investigation.

3) Directly from Blood Cultures

Direct identification of bacteria and yeast from blood culture broth is a promising option for MADLI-TOF MS methods, with the potential to speed the identification process [30, 31]. After preprocessing of the blood culture broth to limit interference from blood cells and hemoglobin and to concentrate the microbes present, the procedure is similar to that used for testing of bacterial colonies.

Identification by MALDI-TOF MS depends on an adequate concentration of the inoculum [32]. Experiments using *Staphylococcus aureus*- and *Escherichia coli*-spiked blood culture broth indicate that bacteria can be successfully identified by MALDI-TOF MS from a culture of 10^7 CFU/mL, whereas the median culture density for a positive identification from a blood culture broth is 10^8 CFU/mL [33]. There is concern that mixed infections would be impossible to identify; therefore, Gram staining would still be required to mitigate that risk. Contrary to most protocols that try to identify infection in a blood culture broth as soon as growth is detected by the automated system [19, 31], one report has proposed that positive vials be maintained for 3-10 hr at room temperature to allow for storage and transport if required [20]. A variety of different protocols have been reported to accurately identify the microorganisms present in positive blood culture broth; however, a lack of standardized protocols, and the use of different software for mass analysis and different bottles for blood culture, makes it difficult to compare the performances of the various methods. One study reported that protocols using extraction are more effective than the IC method [24]. BacT/ALERT vials (bioMérieux) without charcoal were tested, and the authors reported accurate identification with a quick preparation procedure [32]; however, preliminary tests carried out using BacT/ALERT vials with charcoal [34] produced poorer results than those obtained using BACTEC vials (Becton Dickinson), probably due to the presence of charcoal.

In the first large published study of blood culture broth testing,

584 positive blood cultures were tested, and 562 contained a unique bacterial species [31]. Two extraction protocols were used, and accurate results were reported for Gram-negative bacteria at the species level. To accommodate errors in bacterial identification by MALDI-TOF MS when mixed cultures were examined, Gram staining was recommended to optimize detection of individual species. In a study by Prod'hom et al. [19], identification to the species level was obtained for only 79% of 122 positive blood cultures, and identification problems were observed for streptococci and staphylococci, with *Staphylococcus epidermidis* identified only 26% of the time. Stevenson et al. [20] used the Bruker Biotyper 2.0 software to identify 212 positive cultures, and correct identification was obtained at the species level, with scores of <1.9 for 138 isolates (65%), and at the genus level, with scores of <1.7 for 162 isolates (76%). Christner et al. [33] reported accurate identification to the species level (in 95% of 277 samples); of 15 unidentified isolates, three were bacteria for which spectra were not present in the Bruker Biotyper 2.0 database. In a recent paper, the authors confirmed that, in most cases, MALDI-TOF MS accurately identified one of the species present in a polymicrobial culture and produced highly accurate identification at the species level (in 90% of 497 monomicrobial samples). Ferroni et al. [32] described the only study reporting accurate results for blood culture broths of polymicrobial infections; however, this study uniquely used the Andromas database. Vlek et al. [36] reported that the implementation of MALDI-TOF MS in the laboratory resulted in significant improvements to patient care when used for the analysis of positive blood cultures. In their trial, MALDI-TOF MS (with the Bruker Biotyper version 2.0 software) was carried out on blood culture broths. This reduced the time to result by 28.8 hr and increased the proportion of patients receiving targeted antimicrobials within 24 hr of the receipt of the sample by 11.3%. Recently, results were published from the first study to compare two different MS platforms for the direct identification of microorganisms from positive blood cultures; Chen et al. [37] compared the VITEK MS system (bioMérieux) to the Bruker Biotyper MS system (using the version 3.0 software) for identifying microorganisms from 202 positive cultures grown in BACTEC bottles (Becton Dickinson). Sample processing was performed with the Bruker Sepsityper kit, according to the manufacturer's instructions. Identifications by the MS systems were compared to identifications derived from 16S rRNA gene sequencing and a phenotypic method (using VITEK-2). The Biotyper MS system was able to make a higher number of accurate identifications to the species level than the VITEK MS system, and demonstrated better performance than the VITEK MS system with regard to Gram-positive bacteria at the genus and species levels, especially for the identification of *Bacillus* species; both systems performed poorly when analyzing polymicrobial specimens.

The direct identification of yeast isolates from positive blood culture broth has also been evaluated by a number of groups. Two studies with a limited number of isolates, 20 and 18, demonstrated that the identification of yeasts is possible when using samples directly taken from blood culture bottles [24, 32]. However, the accuracy greatly differed between these two studies, with correct identification of 5% and 100% of isolates, for the studies using 20 and 18 isolates, respectively; it is likely that the different protocols, software, and databases used to perform data analysis explain this discrepancy. In 2010, an evaluation of species-level identification of Candida isolates from positive blood culture broth was reported. In their report, Marinach-Patrice et al. [38] noted that a direct identification from the positive broth, bypassing subculture and subsequent identification steps, allowed results to be obtained up to 3 days sooner. The method was tested on one routine positive blood culture from a patient, with a correct identification being obtained, allowing the authors to conclude that the method for the direct identification of *Candida* species from blood culture broth using MALDI-TOF MS was a rapid and accurate mechanism that could lower costs and hasten appropriate antifungal therapy. With regard to the direct identification of yeast, Spanu et al. [39] evaluated the Bruker Biotyper system (using version 2.0 software), for the rapid identification of *Candida* species causing bloodstream infections, in a large routine setting. They were unable to reliably identify the polyfungal isolates that were analyzed, as many users of the Biotyper system have also reported for polybacterial infections. Most importantly, 80% of the positive blood cultures included in their study were identified as positive 24 hr after sample entry, in many cases allowing species-level identifications to be reported to physicians within 24 hr of blood draw [39].

4. Current Limitations

It is important to mention the current limitations associated with MALDI-TOF MS. The outgrowth of microorganisms from

http://dx.doi.org/10.3343/lmo.2015.5.4.176

potentially contaminated material to produce isolated colonies is still required, as the technique's ability to resolve mixtures of microorganisms is lacking. Additionally, a high number of bacterial cells are still required for identification, such that a whole intact colony is typically used for analysis, limiting the ability to rapidly identify microorganisms directly from biological fluids where the bacterial count is expected to be relatively low. Research is currently being carried out to mitigate these limitations. In addition, until accurate determinations of drug-resistance factors can be made, parallel culture-based recovery of positive blood cultures will most certainly be required for the foreseeable future for antimicrobial susceptibility testing. According to Bizzini et al. [12], for 133 strains (corresponding to 85 different species) of 410 strains tested, failure to obtain an identification (score of <1.7) can be attributed to two causes: (1) absence of an adequate reference spectrum in the Biotyper database (58.6% of strains); failure to obtain a sufficient protein signal in order to build a spectrum that can be compared to the Biotyper database (41.4% of strains). Failure to obtain a spectrum can be explained by either the structural properties of the cell wall of some types of bacteria (Gram-positive bacilli being a prototypical example) or the fastidious growth of some isolates, which yield only small amounts of colonies that can be harvested for protein-extraction purposes. La Scola et al. [40] reported that, for anaerobes, MALDI-TOF MS allowed identification of 332 (61%) of 544 isolates, including 100% of isolates identified as *Clostridium perfringens* or *Bacteroides fragilis* and other common *Bacteroides* spp., while, identification levels were above 50% for *Propionibacterium* spp., *Fascioloides magna*, most *Fusobacterium* spp., and *Prevotella* spp.

MALDI-TOF MS is increasingly applied to taxonomic issues in microbiology, e.g., to rapidly reveal cryptic species in large batches of related isolates [41]. However, at present, it is impossible to define a concrete value for the similarity of mass spectral fingerprints of conspecific isolates, i.e., a threshold that separates species. However, this is not entirely surprising because the rules for defining microbial species do not apply to all taxa. Furthermore, historical biases exist, which have defined microbial species according to criteria that are not necessarily correlated with current views of how microbial species should be delineated. When mass spectral fingerprints are analyzed for multiple, well-characterized strains of closely related specie, e.g., by cluster analysis, separation of distinct groups is usually evident. The studies reported in this special issue consistently demonstrate the ability of MALDI-TOF MS to delineate strains at the species-level for several genera, including *Acinetobacter*, *Legionella*, *Leuconostoc*, *Staphylococcus*, *Stenotrophomonas*, *Streptococcus*, and *Yersinia*. Therefore, thresholds of mass spectral fingerprint similarity for discriminating species must be expressly set for the particular taxa. As with DNA sequence data, e.g., 16S rRNA gene sequences, species assignment by mass spectral fingerprinting is not always straightforward. A major reason for this is the intraspecific variation within microbial taxa; while a set of strains of one species could share more than 75% of MS mass signals, this value can be lower than 50% for strains of other species.

Mass spectra-based identifications of individual strains may be hampered by their background similarity and thus only allow assignment to the level of a species complex. On the other hand, mass spectral similarity of approximately 20% has to be considered as non-specific, as demonstrated by the *E. coli* outgroup; with only slightly higher mass spectral similarities, all strains of *Lactococcus* spp. are clearly separated from strains of *Streptococcus* spp., consistent with the taxonomic separation of the two genera [42], although they do not form a genus-specific cluster. When examining the duplicate analyses of individual strains in a dendrogram, a particular spectrum-to-spectrum variation is evident, with mass spectral similarity as low as 70% observed between replicate measurements in some cases. The variation between individual replicate spectra can be reduced, to a large degree, by standardizing the sample-preparation procedures, although a residual "haziness," intrinsic to the MALDI-TOF MS analysis itself, cannot be completely eliminated. In fact, replicate mass spectra with 100% similarity are not practically achievable [43]. Therefore, it can be concluded that advances in sample preparation and new approaches to data analysis have the potential to increase the level of resolution at which MALDI-TOF MS analyses may be applicable. The advantages in terms of the speed and cost of analyses provide a strong incentive for further developments and improvements for extending the range of MALDI-TOF MS applications.

5. Applications for DNA Analysis

Many high-throughput single-nucleotide polymorphism (SNP) genotyping technologies are currently available. Each offers a unique combination of scale, accuracy, throughput, and cost.

However, SNP genotyping is in a state of flux, with no single technology or platform able to satisfy all users and study designs. Certain platforms, such as the Sequenom MassARRAY system, has several attractive features for users desiring an accurate custom-SNP-genotyping assay [44] with modest multiplexing and minimal assay setup costs, due to the use of unmodified oligonucleotide primers. The system utilizes a homogeneous reaction format, with a single extension primer to generate allele-specific products with distinct masses, multiplexed PCR reactions, a single termination mix and universal reaction conditions for all SNPs, small reagent volumes, and a throughput of >100,000 genotypes/day.

The most frequently used methods for genotyping are based either on fluorescence or MS of genotype-specific base-extended primers. MS directly measures the molecular mass of the PCR products, whereas other technologies only indirectly assess PCR products, either through hybridization or DNA sequencing. Procedures using PCR products as templates to which oligonucleotide primers are hybridized, extended, and then analyzed by MS have been widely used [45]; however, these fail to employ one of the key strengths of MS, the direct mass analysis of PCR products. Assays based on hybridization have also been used as surrogate genotyping methods [46, 47], but they are labor intensive. They are also unsuitable for the identification of genotype variants or genotype mixtures, and for screening large numbers of samples, because of the complex protocols involved [48]. A major drawback of these approaches can be the requirement for multiple primers or probes that overlap one another, or the requirement for subtle and complex assay-optimization processes for analyzing a quantity of variations in close proximity. The restriction fragment mass polymorphism assay is based on PCR amplification and precise mass detection of oligonucleotides excised by *Fok*I and *Bts*CI (or other TypeIIS restriction enzymes) with genotype-specific base variations [49]. This assay represents an improvement over previous methods because the mass of PCR products is determined directly, rather than their identity being interpreted on the basis of fluorescent or radioactive reporter tags. Both DNA strands can be analyzed in parallel [49, 50], providing a level of internal confirmation not achievable by other methods. The use of a TypeIIS restriction enzyme makes the assay independent of restriction sites within the human papilloma virus genome, and suitable for many different viral genotypes because these enzymes cleave DNA at a fixed distance from the recognition sites incorporated into the PCR primers.

6. Closing Remarks

Although the methods described above are currently restricted to a few clinical or research laboratories, their potential that moving them into routine clinical microbiology and public health laboratories seems essential. The implementation of MS in the routine clinical microbiology laboratory will provide a powerful and accurate tool to quickly identify bacteria, mycobacteria, and fungi from cultures. Further improvements in specimen processing of blood culture broth and urine samples will be required prior to implementation, as clinical laboratories will be faced with the challenge of selecting between MALDI-TOF MS methods and emerging molecular methods to identify bacteria from broth or directly from specimens. Improvements to spectral databases and analysis software should optimize the use of MALDI-TOF MS methods, and should reduce the turnaround time for identification of nearly all clinically relevant microbes.

요 약

지난 10여 년에 걸쳐, 임상미생물분야는 미생물 동정에 있어 다소 느린 전통적인 동정 알고리즘에서 신속히 결과를 확인할 수 있는 분자진단학적 방법 또는 질량분석법으로의 혁신적인 변화를 겪고 있다. 일찍이, 질량분석법은 임상화학검사실에서의 단백기반 시료 분석법으로 적용되어 왔다. 최근, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) 원리를 이용한 질량분석법이 미생물검사실에 도입되면서 미생물 동정에 있어 신속, 정확한 미생물 동정결과를 제공할 수 있게 되었다. 널리 알려진 제조사의 제품으로 2가지 장비 플랫폼이 기존의 표현형을 통한 동정 자동화 장비를 대체하고 있으며, 일부 경우에서는 염기서열 기반 동정기법 대신으로도 사용되고 있다. 이 종설은 임상연구나 임상미생물검사에 있어 MALDI-TOF 질량분석법의 현재 상황을 요약하고, 시료 처리, 분석 알고리즘, 해석 및 한계를 분석한 최근 연구들을 소개하고자 한다. 또한, 현재 상용화된 MALDI-TOF 질량분석기와 그 분석 소프트웨어에 대해서도 다룰 것이다. 마지막으로, 임상검사실에서 이러한 신규 검사법들의 잠재능력을 개발하기 위해 끊임없이 노력하고 개발자들과의 협력관계를 공고히 한다면 MALDI-TOF 질량분석법을 비롯한 새로운 기술에 있어 향후 진단분야에서의 용도를 새롭게 정의할 수 있을 것이다.

http://dx.doi.org/10.3343/lmo.2015.5.4.176

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article were reported.

ACKNOWLEDGEMENTS

This study was supported by Grant no. 06-2011-056 and 02-2014-050 from the SNUBH Research Fund, and this work was supported by a Research Resettlement Fund for the new faculty of Seoul National University.

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