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Review

MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory

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ABSTRACT

Since the early 1980s, mass spectrometry has emerged as a particularly powerful tool for analysis and characterization of proteins in research. Recently, bacteriologists have focused their attention on the use of mass spectrometry (MS) for bacterial identification, especially Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF). Moreover, recent publications have evaluated MALDI-TOF in microbiology laboratory for routine use. MALDI-TOF-MS is a rapid, precise, and cost-effective method for identification of intact bacteria, compared to conventional phenotypic techniques or molecular biology. Furthermore, it allows identification of bacteria directly from clinical samples (blood cultures for example).
The goal of this review was to update recent data concerning routine identification of microorganisms by MALDI-TOF in the clinical microbiology laboratory.

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The management of patient suspected of bacterial infection traditionally relies on two major tracks, the first one aims to identify the pathogen at the infection site while the second is to find the best therapeutic option using an empirical antibiotic treatment knowing that an adapted antimicrobial treatment can reduce morbidity and mortality [1,2].

The classic strategy for bacterial identification is based initially on fast and simple tests like Gram staining, catalase and oxidase tests. Secondary phenotypic tests complete the identification [3,4]. This identification can be performed using either commercial kits such as miniaturized biochemical tests (API analysis) or automated systems. API analysis takes several hours and in some cases can be imprecise in assigning bacteria to its species. Phenotypic markers for bacterial typing might show variability due to environmental changes such as the conditions of culture. Although some of these tests are performed within minutes, complete identification needs about 18 h after culture in a large numbers of cases or even more for fastidious organisms. Since antibiotic susceptibility testing is conducted in parallel, the resistance phenotype can help to interpret the results of the identification. This approach often requires pure culture of the bacteria and identification is achieved in the best case 48 h after receiving the clinical sample. This time is greatly increased if growth of the organism is slow and/or difficult and if the resistance phenotype is useless.

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Alternatively, molecular biology enables rapid bacterial identification using polymerase chain reaction (PCR), which is one of the most sensitive test. Most PCR used for bacterial identification target conserved genes such as those coding for ribosomal RNA [5,6], RNA polymerase (*rpo*B) [7], or elongation factors [8]. Molecular biology presents numerous advantages. First, PCR theoretically permits identification of slow growing organisms and has been used to establish pathogenesis for uncultivable organisms [9]. Second, results are generally obtained in a short time especially if real-time PCR is used. Unfortunately, the information obtained is not always sufficiently discriminating to obtain identification at the species level. Thus additional steps aiming to amplify other target genes are then required. These molecular biology-based identification techniques require high level of technical expertise, remain cost expensive, and are therefore not suitable for routine identification. Other techniques such as using DNA chips or *microarrays* have also been implemented; however, cost and workload requirements currently preclude their routine use [10].

New approaches are required for rapid analysis of bacteria in clinical microbiology laboratories to improve care of patients. Among recent development for bacterial identification, the use of protein profiles obtained by MALDI-TOF-MS directly from colonies was successfully proposed. The method analyzes the profiles of bacterial macromolecule that are obtained from whole bacteria. This new proteomic approach allows rapid and accurate identification of bacteria as well as yeast and fungi.

This review analyzes recent publications for routine identification using MALDI-TOF-MS on intact microbial cells and directly from samples such as blood cultures.

MALDI-TOF-MS

The intrinsic property of mass spectrometry is to detect the mass-to-charge ratio (m/z) of a bioanalyte, providing spectra within minutes. The method has been used to profile bacterial proteins from cell extracts and has recently been applied to the identification of microorganisms from different genera, different species, and from different strains of the same species. The procedure provides a unique mass spectral fingerprint of the microorganisms. This method requires that the biopolymer molecules normally present in the condensed phase be converted into intact, isolated ionized molecules in the gas phase. Then, ions are separated according to their molecular weight after migration in an electric field. Each molecule detected is characterized by: the molecular mass (m), the charge (z), the ratio mass/charge (m/z), and the relative intensity of the signal. The applications of mass spectrometry are very large, comprising highly accurate analysis of peptides and determination of peptide sequences to identify and characterize the state of proteins in biological sample [11].

In early experiments, only molecules of low molecular masses were analyzed. The limit size varies from 1000 dalton (Da) for biopolymers to 9000 Da for some synthetic compounds [12]. Soft ionization techniques such as MALDI-TOF [13,14] and electrospray ionization (ESI) [15], which were introduced in the late 1980s, have largely overcome the problem of harsh ionization. Of these two techniques, MALDI-TOF proved to be most effective for bacterial identification. Indeed, it allows the detection of macromolecules in complex mixtures without prior purification of samples [16]. The first step is the formation of a crystal between the sample and an organic matrix (co-crystallization). The sample is spotted onto a MALDI-TOF sample target with an appropriate matrix and allowed to air dry at room temperature. Then, the plate is inserted into the MS, the dried matrix-sample mixture is bombarded with a laser to create gas phase ions that are then pulsed into a flight tube. Generally, only a singly ionized species having a single charge is produced. The species of interest are identified by their mass/charge ratio, the m/z value is obtained from the centroid of the peak. Initial tests using this technology were able to generate a large number of intact molecular ions as well as dimers of proteins in the mass range above 10,000 Da, for example bovine albumin (67,000 Da) [13].

The detection of mass spectral fingerprint has become a convenient tool for the rapid analysis of bacteria. The method analyzes the profiles of bacterial components that are extracted from intact bacteria. The first report proposing bacterial identification based on MALDI-TOF analysis was by Holland et al. in 1996 [17]. Unlike previous studies, the bacteria were not undergoing any treatment before the analysis. The same year, Krishnamurthy et al. reported similar results of bacterial identification by MALDI-TOF. They obtained spectral fingerprints of pathogenic species such as *Bacillus anthracis*, *Brucella melitensis*, *Yersinia pestis*, and *Francisella tularensis* [18]. Ever since, the number of publications concerning bacterial but also mold and yeast identification increases exponentially.

Databases

The identification by MALDI-TOF is based on the following findings: (i) spectral fingerprints vary between microorganisms (Fig. 1), (ii) among the compounds detected in the spectrum, some peaks (molecular masses) are specific to genus, species, and sometime to subspecies, (iii) spectra obtained are reproducible as long as the bacteria are grown under the same conditions.

The same species can give different mass spectra, owing to use of different growth conditions or different chemical extraction methods. Therefore, well-controlled growth conditions and standardized sample preparation procedures are crucial to obtain reproducible mass spectra. The solution composition used to harvest the bacteria can modify the spectra of a given strain. For example, the use of trifluoroacetic acid or formic acid creates different spectra with significant differences in relative intensities of the peaks. Methods of protein extraction, concentration of NaCl, and spotting methods [19,20] can influence the quality of the spectra by modifying the crystallization of the sample with the matrix [21]. The nature of the matrix is one of the most important parameter affecting the quality of the spectrum [22]. The matrix is believed to serve two major functions: absorption of energy from the laser and isolation of the biopolymer molecules from each other. A great number of different matrices are available. They all require physical and chemical properties: (i) an efficient absorbance at the laser wavelength, (ii) an efficient ionization, (iii) an important stability not to interfere with the mass spectrum of the sample. The choice of the matrices depends on the nature of the sample studied. The matrices most commonly used are 2,5-dihydroxybenzoic acid (gentisic acid), 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and α-cyano-4-hydroxycinnamic acid (α-CHCA). Gentisic acid (DHB) allows the study of oligosaccharides, glycopeptides, and glycoproteins. Generally, the DHB is more efficient for small molecular weight components and sinapinic acid and CHCA especially allow the study of proteins [23]. The use of ferulic acid as matrix allows the study of high molecular weight proteins, up to 70 kDa [24]. Other parameters influence the crystallization (thickness and consistency of the dried sample spot) [25]. For the same species, mass spectral fingerprints are different depending on the matrices used (Fig. 2). These observations stress the need for careful attention in the preparation of the sample to obtain optimum reproducibility.

MALDI-TOF-MS is based on the analysis of phenotypic characters, which vary with culture media and incubation times. Several studies have investigated the impact of growth culture conditions and all have found variations in spectral fingerprints. However, identification remains possible [26,27]. Incubation time also influences the quality of spectra [20,28]. However, when experimental and environmental conditions are controlled, the technique is reproducible [20,25,29].

Finally, several studies dealt with the differences observed for the same sample analyzed with two different mass spectrometers

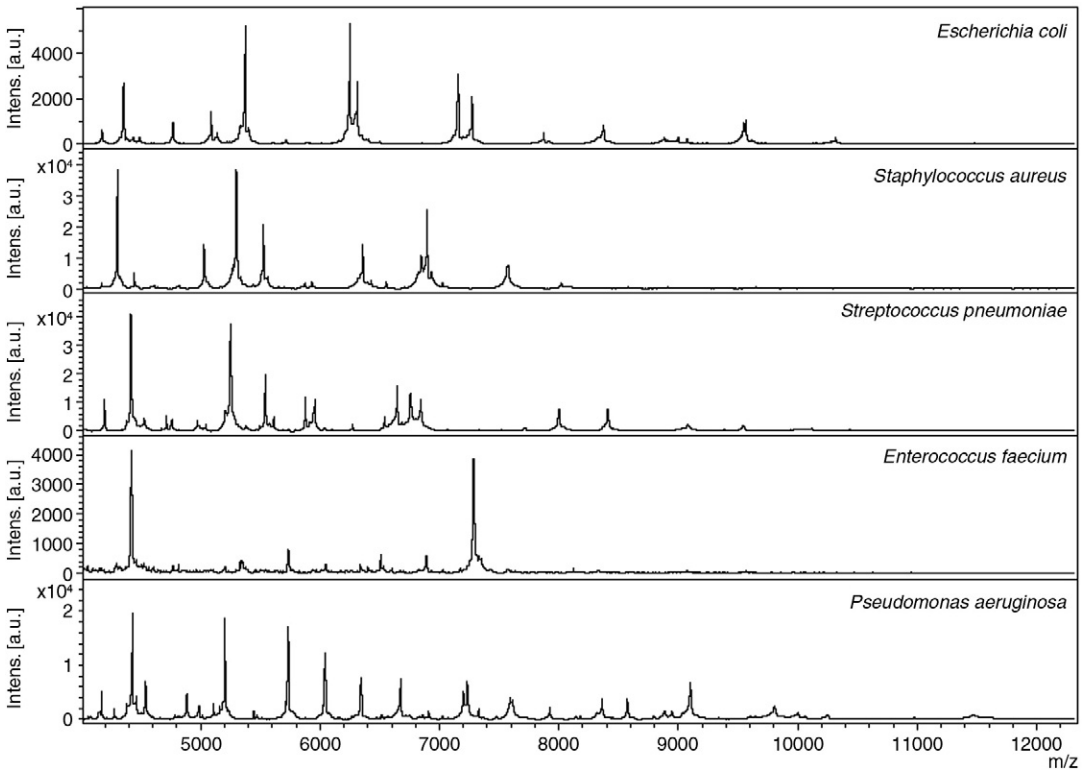

Fig. 1. Spectral fingerprints obtained from whole colonies of five different bacterial species. The matrix used is HCCA.

[21,30]. For Walker et al. spectra were very close, with an overlap of 60% of the peaks, most of the differences relating to their relative intensity [27]. Despite significant variation of mass spectral pattern, which result from changes in experimental conditions, many peaks remain conserved. These conserved peaks represent the ones that have the best potential for use as biomarkers for bacterial identification. Nevertheless, when optimization of sample treatment/analysis methodologies is achieved, it becomes possible to identify with confidence species-, genus-, and strain-specific protein biomarkers of bacterial spectra.

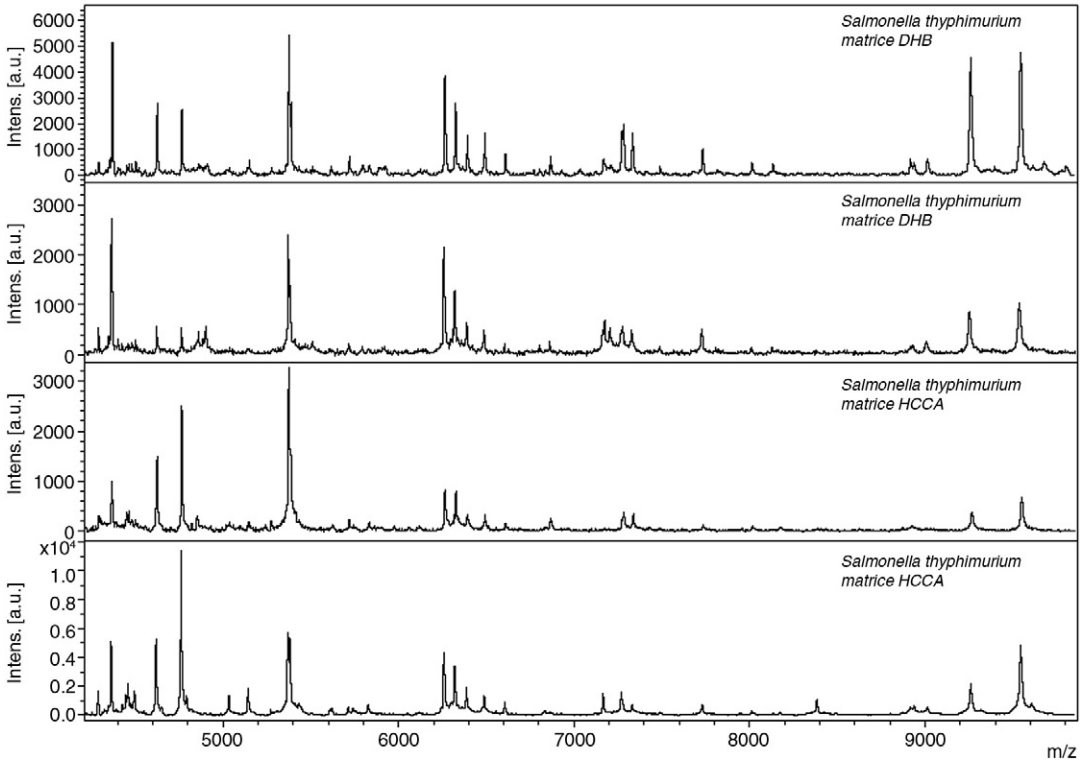

Fig. 2. Spectral fingerprints of the same strain of *Salmonella typhimurium* obtained with two different matrices: DHB and HCCA. The two top spectra correspond to two repetitions with DHB and the two bottom spectra of two repetitions with HCCA. This figure illustrates the variations observed depending on the matrices used onto distinct acquisitions.

Two main strategies have been developed to engineer databases. The first one consists of engineering a large database in which a large number of peaks are retained for each reference strain, not focusing on a limited number of peaks, which may be species specific. This strategy requires the use of several reference strains for each species that needs to be included in the database. When a given strain is tested, the species retained for the tested strain is that of the species of the reference strains having the best match. The second strategy retains for each species, a limited number of peaks having a high intensity and conserved after several assays on a limited number of strains representative of the species. These peaks are therefore likely to be specific peaks resulting in a spectrum that is searched for when a new strain is tested. The latter databases encompass a limited amount of data and are likely to be less influenced by the growth conditions of the tested strain [31].

Identification by MALDI-TOF in routine medical microbiology laboratory

Intact bacteria

Early studies could identify a limited number of bacteria, often two different species. Actually, different systems (MALDI-TOF and databases) allowing identification of the main microorganisms (bacteria, yeasts, fungi) isolated from clinical samples are available. A new area has begun with the replacement, already effective in some laboratories, of conventional systems by MALDI-TOF-MS. All the following studies have been conducted with acquisition directly on whole bacteria obtained on Petri dishes.

For the first time, Seng et al. describes the use of MALDI-TOF in routine clinical microbiology. A large number of strains were studied: 1660 strains (45 genera, 109 different species with 1 to 347 isolates per species) [32]. This assessment focuses on the performance of MALDI-TOF in terms of identification. A single colony is directly deposited on the MALDI-TOF target plate and 4 such deposits are made for each isolate. The identification is considered valid if two or more deposits provide the same identification with scores acceptable for validation. This approach is particularly interesting because it illustrates the way to circumvent the variability described above. Only 260 isolates (15.7%) did not yield an accurate identification after reading of 2 spots. For these isolates, identifiable profiles were obtained after reading the two remaining spots. According to the authors, one or both spots were either empty or too small to allow a correct analysis. This study confirms the excellent results obtained by this technology with more than 95% correct identification: 84% at the species level and 11% at the genus level. In 46 cases (2.8%) strains were not identified by MALDI-TOF, and in 28 cases (1.7%), the identification was erroneous, despite a high score allowing result validations. The main difficulties were observed with streptococci, among which *Streptococcus pneumoniae* and *Streptococcus mitis* that are related species giving close spectra. Misidentification of these strains may have clinical consequences if ignored. More surprisingly, correct identifications of staphylococci were lower than those obtained in previous studies [31,33–35]. Erroneous identifications were obtained for some strains of *Stenotrophomonas maltophilia*, *Propionibacterium acnes*, and *Shigella* sp. For *P. acnes*, the authors hypothesized that the unique spectrum may not be representative of the true diversity of *P. acnes* profiles, and the inclusion of additional *P. acnes* spectra in the database may improve correct identification. They have also estimated that the average time to transmit the result to the physicians is less than 10 min, and 3 to 5 times less expensive compared to conventional identification systems. The authors did not observe any discrepancies between MALDI-TOF-MS and Gram staining, suggesting that MALDI-TOF-MS could be used as a first line without prior Gram staining. These results confirm the role of MALDI-TOF in laboratories, but they also stress the importance of updating databases to fill certain gaps or to improve the identification of some species especially *S. pneumoniae* [32].

For van Veen et al. the experimental approach is similar to Seng et al. with two spots per sample followed by an extraction step if no identification was obtained. Correct identification was obtained for more than 97% strain (92% at the species level and 5.1% at the genus level). In this work, yeasts were tested (61 isolates spread over 7 genera and 12 species), 85.2% were correctly identified at the species level, and 96.7% at the genus level, demonstrating the effectiveness of this technique for these microorganisms. Again problems were encountered for discrimination between the viridans streptococci group and pneumococci, as well as for anaerobic bacteria. The authors also emphasize that the database need improvement with more spectra of well-characterized streptococcal species [36].

In the study of Blondiaux et al., only 264 (73%) of 362 strains analyzed were identified at the species level. Their conclusions are identical to the previous studies with difficulties to identify viridans streptococci and pneumococci as well as HACCEK, *Shigella* and strictly aerobic bacteria (*Aeromonas* spp., *Achromobacter* spp, *Alcaligenes* spp.). The authors rightly stress the importance of the number of strains in the database and the lack of completeness for some species. Furthermore, they propose the creation of a Committee of Experts for the control of update to avoid irrelevant entries [37].

Direct detection of microorganisms from sample

Several attempts have been made using molecular biology and especially the real-time PCR to identify bacteria from positive blood cultures detected [38,39]. Rapid methods using microarrays, hybridization probes, and even techniques of flow cytometry have recently been reviewed for diagnosis of bloodstream infections [40].

Recently, several studies have evaluated the contribution of MALDI-TOF for the identification of microorganisms in positive blood culture broths (for recent review, see Drancourt [41]). The important first step consists to separate the bacteria from cellular components in absence of which no identification is obtained [42]. Whatever is the protocol used, different centrifugation steps are needed followed by lysis of blood elements. For these studies, the percentages of correct identification at the species level varied from 31.8% to 95% depending to Gram positive or negative species and to the protocol used (Table 1) [42–47]. Different hitches were encountered: correct identification of viridans streptococci and *S. pneumoniae* in the presence of polymicrobial blood culture broths, in the best case only one species was identified. These results pointed out the importance of Gram straining to verify the presence of one or more species. Ferroni et al. have made a significant progress in sample preparation [45]. Once the blood culture is detected positive using a mild detergent to lyse the cellular membranes [42–44], they could decrease this step down to few minutes allowing identification in less than 30 min. MALDI-TOF-MS becomes therefore the fastest of all techniques for bacterial identification directly from blood culture broths, thus allowing a real-time diagnosis of bloodstream infections.

In a recent study, Ferreira et al. have evaluated direct identification of bacteria from urine samples by MALDI-TOF. Two hundred and sixty urine samples, detected as positive by the screening device (flow cytometry UF-1000i, bioMérieux), were processed by both culture and MALDI-TOF. Like positive blood culture broths, different centrifugation steps are needed before applying samples directly to the MALDI-TOF plate. Twenty samples were also negative in culture and MALDI-TOF. Overall, correct identifications were obtained at species and genus levels for 79.2% and 80%, respectively. MALDI-TOF MS seemed to require high bacterial count to be able to afford reliable score. In fact, among the 220 microorganisms causing urinary tract infections with bacterial growth $>10^5$ CFU/mL, correct identifications at species and genus levels were 91.8% and 92.7%, respectively. For *E. coli*, the most frequent bacteria isolated in urinary tract infections,

Table 1
Summary of major studies using MALDI-TOF for bacterial identification. GN: Gram negative, GP: Gram positive.

Authors	Sample		Id species level	Id genus level	Main identification difficulty	Comments
Seng et al. [32]	Routine ($n=1660$)	all routine samples	83.8%	95%	*Propionobacterium acnes*, *Streptococcus pneumoniae*, *Stenotrophomonas maltophilia*, *Shigella* sp.	First line method of identification
van Veen et al. [36]	Routine ($n=980$)	all routine samples	92%	98.8%	*Streptococcus pneumoniae*, anaerobic bacteria	
Blondiaux et al. [37]	Routine ($n=362$)	all routine samples	72.9%	87%	viridans streptococci group. *Shigella* sp.	
Prod'hom et al. [42]	Blood ($n=126$)	positive blood culture	77.8%, GN: 89.1%, GP: 71.6%	78.7%, GN: 89.1%, GP: 72.9%	*Streptococcus mitis* group, *Staphylococcus* sp.	The presence of a capsule explain partially the low identification rate of *S. pneumoniae*, *H. influenzae*, *K. pneumoniae*
La Scola et al. [43]	Blood ($n=599$)	positive blood culture	76%	76%	*Streptococcus* sp., polymicrobial samples	
Stevenson et al. [44]	Blood ($n=212$)	positive blood culture (179), spiked bottles (33)	80.2%	80.2%	*Streptococcus mitis* group, *Propionobacterium acnes*	
Ferroni et al. [45]	Blood ($n=685$)	positive blood culture (388), spiked bottles (312)	89%	98%	*Streptococcus pneumoniae*, *Streptococcus mitis* group	For mixed culture, most abundant germ was in most cases identified. Fast method
Christner et al. [46]	Blood ($n=277$)	positive blood culture	94.2%	95%	Cocci Gram +	Mismatching mostly resulted from insufficient bacterial count and occurred preferentially with Gram +
Ferreira et al. [47]	Blood ($n=300$)	positive blood culture	42.6%, GN: 83.3%, GP: 31.8%	71.6%, GN: 96.6%, GP: 65.7%	*Streptococcus mutans*, *Staphylococcus* sp., *Staphylococcus aureus*	No mixed culture
Ferreira et al. [48]	Urine ($n=220$)	positive urine samples	91.8%, GN: 93.6%, GP: 66.6%	92.7%, GN: 94.6%, GP: 66.6%	*Streptococcus* sp., *Enterococcus* sp., *Raoultella* sp.	Best results with high bacterial account $>10^5$ CFU/mL, *E. coli* $>10^5$ CFU/mL: 97.6% correct id rate, 5 mixed cultures: 3 identifications

correct identification was obtained for 97.6% if the colony count was $>10^5$ CFU/mL. In case of mixed culture (5 samples), MALDI-TOF provided no identification in two, but reported correct identification in 3. In these cases, correct identification was probably dependent on the proportion between both populations [48].

Outlook and development

MALDI-TOF-MS will soon become a widely used technique in routine clinical laboratories for bacterial identification replacing automates and other phenotypic techniques. A great addition to its routine use for identification would be the detection of antibiotic resistance associated to the identified bacteria. Several teams have attempted to differentiate strains of methicillin-resistant *Staphylococcus aureus* (MRSA), which harbor the *mecA*, gene from methicillin-sensitive strains (MSSA) [16,27,49–51]. Edwards-Jones et al. studied 14 *S. aureus*, 7 MRSA, and 7 MSSA. They showed that spectra of MRSA contained more peaks (82 to 209) than those of MSSA (37 to 67). Some peaks were specific to MRSA, others were specific to MSSA, and some were specific to individual strains. However, two strains of MRSA were not correctly identified [49]. According to Bernardo et al., analysis of clinical MRSA by MALDI-TOF does not give a specific MRSA profile but allows strain to strain differentiation among patients [50]. In the study by Du et al., 76 MRSA and MSSA strains were analyzed. Thirty-three strains had *mecA*, as detected by PCR and were identified as MRSA by MALDI-TOF; 36 were negative by PCR and identified as MSSA by MALDI-TOF; 7 were negative by PCR but identified as MRSA by MALDI-TOF [16]. Finally, Walker et al. compared 14 spectra of MRSA to 6 spectra of MSSA. They highlighted differences between the spectra of these two types of strains, but they did not propose a specific spectra for MRSA identification [27].

Research and detection of particularly virulent strains highly concerned physicians since identification of strains producing some virulence factors may be of help in the management of infections. Panton-Valentine Leukocidin (PVL) is a prime candidate. In the study by Bittar et al., a marker (m/z 4448) has been shown to differentiate between strains of *S. aureus* producing PVL and those who do not [52]. This approach appears promising but as for detection of antibiotic resistance lot of works remain to be done before it can be used in clinic.

In conclusion, identification by MALDI-TOF-MS is effective in identifying bacteria but also yeast and fungi, and several studies have shown that its use is well suited for identification in routine microbiology laboratory. It is also currently the fastest technique to accurately identify microorganisms grown in positive blood culture broths. Since comparison between the MALDI-TOF and in particular the identification systems attached to it have not been thoroughly performed double-blind studies involving several laboratories seem particularly important to compare and improve the various MALDI-TOF and databases commercially available.

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