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Is MALDI-TOF Mass Spectrometry a Valuable New Tool for Microorganisms Epidemiology?

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Abstract

Matrix-assisted laser desorptionionization time of flight mass spectrometry (MALDI-TOF MS) is now accepted as a fast and reliable method for bacterial identification. It has been shown a very accurate method for identifying bacteria and yeast and moulds from culture, but also from blood cultures and directly from some samples, such as urine. Some authors have proposed the application of the protein profiles obtained by MALDI-TOF MS for bacterial typing and hierarchical clustering with epidemiological purposes.

Here we revise and discuss the information published in the last years on the possible usefulness of MALDI-TOF MS for this purpose, the approaches proposed for different microorganisms and the future perspectives of this technology in the area of infectious diseases epidemiology.

Keywords: MALDI-TOF mass spectrometry; Bacterial epidemiology; Molecular epidemiology

Introduction

Mass Spectrometry (MS) is an analytical technique based on the separation and detection of particles. The first applications of this technology were in routine chemical analysis of hydrocarbon mixtures in the 40 s. Thereafter, it has been used to elucidate the structure of organic compounds such as polypeptides, proteins and high molecular weight biopolymers.

Gradually, MS was consolidated as a qualitative and quantitative analytical technique, which allowed the accurate identification of a wide range of compounds [1].

The use of MS in microbiology was first described in 1975, when Anhalt and Selau [2] used this technique for the identification of bacteria. The application of MS in microbiology improved in 1988, when the Matrix-Assisted Laser Desorption Ionization (MALDI) method was described as an ionization method useful for large biomolecules [3], since the matrix allows the sample crystallization and subsequent ionization uniformly, without degrading. In MALDI MS, the source of desorption (the laser beam) falls upon an alcoholic acid solution of the sample mixed with an excess of a matrix substance, which is responsible for absorbing radiation and transferring the energy to the sample. It converts the nonvolatile solid or liquid samples in gaseous ions. The choice of the matrix is crucial to the success of the experiments. Cinnamic acid derivatives, such as the α-cyano-4-hydroxycinnamic acid, are readily soluble in organic solvents such as acetonitrile and trifluoroacetic acid. Its slow evaporation allows the sample crystallizing evenly. Proteins are better distributed in these crystals, being confined directly on the surface of the probe [4].

The ions generated, usually single charge (positive), undergo the action of a high electric potential, thus acquiring a kinetic energy. Most ions are single charge, and the remaining parameters remain constant, thus the mass/charge (m/z) ratio is usually the ion mass. Since all ions have the same kinetic energy, their speed will vary inversely proportional to their masses. Therefore, larger particles will migrate more slowly than smaller particles. In the mass analyzers *Time-of-Flight* (TOF), a pulsing electric field accelerates the ions produced, and separation according to their mass occurs during its route through a vacuum tube to a detector located at the end of this tube. The computer controlling the mass spectrometer receives the signals generated by the detector and generates a spectrogram, that is, a graph where the relative abundance of the peaks of each ionic species is represented with respect to their mass/charge ratio.

The main advantage of this method is obtaining very simplified spectra from high molecular weight, thermally unstable compounds, such as proteins. This allowed the introduction of MS as a powerful tool for environmental and pharmaceutical analysis, food industry and especially in the study of complex proteins involved in biological processes, which eventually could be used as disease biomarkers [4] and applied to microbial identification [5]. Its accuracy and sensitivity, and its ability for desorption of thermally labile, high molecular weight molecules, combined with a wide range of mass detection, make it a suitable method for determination of biomolecules such as peptides, proteins, oligosaccharides and oligonucleotides [6].

MALDI-TOF MS and Microorganisms Identification

Matrix-Assisted Laser DesorptionIonization Time of Flight Mass Spectrometry (MALDI-TOF MS) is now widely accepted as a fast and

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reliable method for bacterial identification, which allows identification around 1 day sooner than conventional methodology. It can be applied to biological samples with minimal preparation. The sample is mixed with a low molecular weight compound that strongly absorbs laser light (matrix), and the dried matrix-sample mix is then exposed to multiple laser pulses, which sublime the matrix and ionize the sample. The charged ions are then accelerated under an electrical field towards a detector, and the time of flight under acceleration allows calculating the mass/charge ratio (m/z) of each peak. The resulting spectrum of m/z peaks can then be matched against a database of reference spectra. MALDI-TOF MS has been shown an accurate method for identifying bacteria, yeast and moulds from culture plates, from blood cultures and even directly from some samples, such as urine [7-16].

MALDI-TOF MS and Bacterial Typing

The protein profiles obtained by MALDI-TOF MS are frequently much more complex than necessary for identification purposes. For identification purposes, the spectrometers use mainly peaks appearing consistently and in moderate to high amounts in a genus or species. Protein peaks appearing inconsistently are less useful for identification, but may be associated to intrinsic characteristics of a group of microorganisms, to below the species level, and therefore could allow defining groups equivalent to those obtained by genetic techniques used in molecular epidemiology [17]. The application of protein profiles obtained by MALDI-TOF MS for bacterial typing and hierarchical clustering with epidemiological purposes has been proposed before [18,19]. However, few studies have been developed so far exploring this possibility, and most of them using different methodologies on different species, which makes comparison difficult.

First Attempts of Typing Bacteria with MALDI-TOF MS

The first studies in which authors tried typing *Staphylococcus aureus* isolates and differentiating methicillin-susceptible and methicillin-resistant isolates by MALDI-TOF MS were published more than 15 years ago [20-22]. Though first results were promising, they have not been confirmed by further studies, thus the usefulness of this method remains uncertain.

The possibility of subtyping *S. pneumoniae* isolates, including non-encapsulated or non-typeable isolates by MALDI-TOF has also been reported [23], but once again there are no further studies that confirm the accuracy of this method. There is also a report [24]) showing that MALDI-TOF MS is able to subtyping *Listeria monocytogenes* isolates, with absolute accordance with the hierarchical clustering obtained by Pulsed Field Gel Electrophoresis (PFGE).

However, other studies developed on other genera, such as those of Tanigawa on *Lactococcus* [25], showed that MALDI-TOF MS is able to identify the different species with a high reliability, but does not subtype them with a level of reliability comparable to Multilocus Sequence Typing (MLST) [25].

Some authors have tried to identify *Salmonella* isolates to the serotype level by using MALDI-TOF MS [26,27]. In a first study in 2003, no definitive conclusions were obtained [26]. Nevertheless, a second study [27] suggested a good enough capacity for classifying genotypes, even proposing specific peaks that shall be considered for some serotypes. The spectra obtained in this second study [27] have more information both in the molecular range and in the number of peaks in each spectrum, probably associated to the optimization of matrix composition obtained with sinapinic acid inclusion. This, together with a better biomarker peak selection, probably contributes to the better results in this study.

MALDI-TOF MS Methods, Parameters, and Bacterial Typing

These studies pose, for the first time, the methodological problems that shall be resolved for obtaining reproducible results. While microorganism identification with MALDI-TOF MS is a very robust method, which does not require usually a meticulous care of study conditions and parameters for obtaining good results, microorganism typing is probably much more demanding in this aspect. Technical parameters concerning microorganisms growing (culture media, growing conditions, etc.) and MALDI-TOF MS preparation and development (matrix concentration and type, sample preparation procedure, matrix plus solvent mixture, acid concentration added to the matrix) may influence the protein profile obtained. In fact, in one of the studies on *Salmonella* previously referred [27], authors report that, for this microorganism, sinapinic acid matrix leads to more complex, and therefore more informative spectra than other matrix.

The software used can also decisively determine the data generated. The existence of so many variables has prevented so far the development of a useful common method. The methods described by now offer satisfactory results only for restricted groups of microorganisms, under very specific conditions, and always with the limitation of the lack of wide enough studies. All these factors, in whole, mean a critical constraint for a more systematic implementation of this methodology. As a result, the prospects raised by researchers regarding the potential usefulness of MALDITOF MS for determining clonal relationships between different groups of microorganisms are very heterogeneous and often contradictory.

Gram Negative Bacteria Typing with MALDI-TOF MS

Acinetobacter spp.

Acinetobacter spp., and especially *Acinetobacter baumannii,* are glucose non-fermenter Gram-negative bacilli, frequently multidrug-resistant, involved in nosocomial outbreaks. These nosocomial outbreaks have direct repercussion on hospital stay length, health care cost and patient mortality. Tracking *A. baumannii* isolates is essential for controlling these hospital outbreaks. The reference tracking methods are molecular methods, which are expensive and time-consuming. Thus, the possibility of using MALDI-TOF MS spectra for obtaining hierarchical classifications similar to those obtained with molecular methods, but much faster and with virtually no additional expends is attractive.

A study published in 2013 on 35 *A. baumannii* clinical isolates, organized in three clusters by Repetitive Palindromic PCR (REP-PCR) [28], showed that MALDI-TOF MS lead to a very similar hierarchical clustering.

By contrast, a second study published recently on 58 *A. baumannii* clinical isolates belonging to worldwide spread clones (ST98, ST103, ST208, ST218) suggest an insufficient capacity of the method to discriminate between microorganisms belonging to different clones [29]. These authors report a great similarity not only between microorganisms belonging to the same clone, but also between microorganisms belonging to different clones, so being unable to find any correlation on the base of the presence/absence of specific peaks, even when they analyzed isolates known as belonging to different genotypes. With the software based on MatLab® used in this study, they were able to predict accurately the clustering of up to 70% of the isolates from some genotypes, such as ST98, but for most genotypes,

accuracy did not reach 50%. Comparing these two studies is difficult, since we have not the same information of all isolates (we know the clones to which isolates belong in one study [29], but not in the other [28]; the software packages used are different). Perhaps the success of Mencacci et al. [28] was due to the use of solid software as Biotyper.

Klebsiella spp.

Tracking isolates is important in enterobacteria, especially in extended-spectrum beta-lactamase or carbapenemase-producing isolates, because of their potential for leading to hospital outbreaks. The reference methods are also molecular techniques, but MALDI-TOF MS might have advantages in terms of quickness and expense. In a study on *Klebsiella pneumoniae* clinical isolates obtained in France and Algeria, published in 2013, and using different software (PASW®), the clones found by using MALDITOF MS data had a good correlation with clinical and geographical origin of the isolates, but not with the types found by Multilocus Sequence Typing (MLST) [30].

Legionella spp.

None of the data mining methods based on MALDI-TOF MS applied seems to offer results comparable to those obtained by PFGE for *Legionella pneumophila* serogroup 1. A study published in 2011 [31], used FlexAnalysis and MALDI Biotyper software (Bruker Daltonics, Germany) and a conventional PFGE procedure for typing 10 *L. pneumophila* type strains, and two groups of environmental isolates from natural water collections geographically distant, with 6 and 7 isolates respectively. PFGE showed different restriction patterns for the type strains, with levels of similarity in the dendrogram lower than 70%, and classified the seven strains of one environmental group and 5 out of 6 of the other into two clusters, leaving one of the latter group with a level of similarity of about 70% with both groups. The dendrogram obtained from MALDITOF MS data by Biotyper software (Bruker Daltonics, Germany) established three clusters. One of them included 8 of the 10 type strains, with a low level of similarity; another cluster included 6 of the 7 environmental isolates from the first group, one of the 2nd group and two type strains, and the third cluster included 5 of the 6 isolates in the 2nd group of environmental isolates and one isolate in the 1st group. Furthermore, the level of similarity within each cluster obtained from PFGE and from MALDITOF MS were very different.

Staphylococcus aureus

A recent study, based on a discretely different approach [32], reports an excellent correlation between the hierarchical clusterings obtained by genetic techniques and by MALDITOF MS in 82 methicillinresistant *S. aureus* (MRSA) isolates from major epidemic strains (CC5, CC8, CC22 and CC398). In this approach, authors select a reference strain of each clonal complex, and identify its protein profile as a reference profile in the identification software. Then, they compare other staphylococci against those "clonal complex type profiles", just as is usually done for conventional identifications. Though it is still necessary to assess the usefulness of this method in other groups of microorganisms, the results support the alternative use of this approach for hierarchical clustering.

Candida spp.

A recent study reports on the potential use of MALDITOF MS both for identification and for hierarchical clustering of more than 150 *Candida* isolates [33]. The authors obtained excellent identification results, which merely confirmed previous reports [34,35]. They used the Biotyper 3.1 software (Bruker Daltonics, Germany) for hierarchical clustering, applying a correlation distance measure method, and an average linkage algorithm. According to this study, the Biotyper 3.1 software classify *C. albicans* isolates in clusters that correlate with the hospitals they were obtained from and with the moment they were isolated. A similar distribution was observed for *Candida parapsilosis*, but not for *Candida glabrata* and *Candida tropicalis*. The main limitation of this study was the absence of a genetic reference method allowing the validation of this hierarchical clustering. Anyway, the good correlation of this hierarchical clustering with the source hospital and the isolation chronology, suggest that it might effectively correlate with an actual epidemiological proximity between isolates. Another recent study [36] on yeasts clustering by using MALDI-TOF MS data showed a remarkable similarity between MALDITOF MS and Microsatellite Length Polymorphism (MLP) hierarchical clustering of *C. glabrata* isolates. In this study, proteins were extracted using the conventional extraction method recommended by Bruker Daltonik GmbH, using formic acid and acetonitrile, and MALDI-TOF MS analysis was performed using the Microflex LTTM system (Bruker Daltonik) with the default settings recommended by the manufacturer. The MALDI-TOF phylogenetic tree was made by using the BioNumerics 7.0 software (Applied Maths NV, Sint-Martens-Latem, Belgium) for generating a dendrogram based on the Pearson correlation coefficient. Moreover, MALDI-TOF MS profiles allowed differentiating fluconazole-susceptible and fluconazoleresistant isolates.

Other microorganisms

MALDITOF MS has also been applied, using the native software by Bruker Daltonics (Biotyper), to type *Ochrobactrum anthropi* [37], a microorganism infrequent as a human pathogen, but increasingly associated to infections related to external devices, probably due to its ability to adhere to silicone. This study also used a correlation distance measure method and an average linkage algorithm, obtaining a hierarchical clustering similar to that obtained by REPPCR, although with some differences whose interpretation is difficult, because the reliability of genetic methods in this group of microorganisms is not exactly known.

Bacteria typing by combining MALDI-TOF MS and PCR

Other authors have used a mixed methodology for hierarchical clustering of microorganisms, so that amplicons obtained by PCR, with the primers commonly used for MLST, are then studied by MALDITOF MS [38]. These authors obtained promising results with *Streptococcus suis*, but the method has not yet been used with other microorganisms. According authors, this method would reduce significantly the time for assay completion, the labor requirements and probably the per-isolate cost, as compared to conventional MLST, particularly when applied to a large number of isolates. This method may be useful, with the limitation that this procedure does not use the conventional MALDI-TOF database used for identification. Instead, a reference library shall be constructed using allelic sequences from the MLST database.

Monitoring of multidrug resistant microorganisms outbreaks

One of the areas where the hierarchical clustering of clinical isolates by MALDITOF MS is being studied is the monitoring of outbreaks caused by microorganisms carrying resistance mechanisms that inactivate specific antibiotics. A study published in 2011 suggested that the performance of MALDITOF MS was lower than that of REPPCR for hierarchical clustering of a group of carbapenemase-producing *Klebsiella* isolates [39]. Another recent study on a group of

carbapenemase-producing *K. pneumoniae* isolates, using a correlation distance measure and a hierarchical clustering based on an average linkage algorithm, was able of classifying isolates into two clonal groups, but the correlation with the hierarchical clustering obtained by MLST was poor [40]. Nevertheless, a recent study reports that MALDI-TOF MS data, processed with data mining methods based on euclidean distance measure and a simple linkage algorithm, allowed to differentiate two clones within a small outbreak of extended-spectrum beta-lactamases (ESBL)-producing *Escherichia coli*, with virtually identical results as PFGE [41].

Bioinformatics methods definition

Defining bioinformatics methods to be used is probably one of the most outstanding issues at this time. A study on *Candida* referred above [33], used an algorithm based on correlation distance measure, and a hierarchical clustering based on average linkage algorithm. The study on ESBL-producing *E. coli* cited in the above paragraph used methods based on euclidean distance measure and a simple linkage algorithm [41], and other studies use different methodologies without any clear criterion on which to use. Some studies even combine different software tools [42]. In a study on *E. coli* hierarchical clustering in different phyla, two strategies were raised: one using a dendrogram created by Biotyper® software (Bruker Daltonics), and another with ClinProTools® software. The former rendered 96% sensitivity and 84% specificity in terms of allocation to B2 phylogroup or to the rest. However, it was not possible to differentiate between phylogroups other than B2. Studies with ClinProTools® allowed a better differentiation between groups other than B2, though the effectiveness did not reach 100%.

Probably, using a common algorithm for any epidemiological application based on MALDITOF MS will not be possible, just as the molecular epidemiology reference methods are not the same for all microorganisms. However, it is important defining stable and predetermined criteria outlining the methodology to be used in each study uniformly and globally.

Clustering of clones harboring specific mechanisms of pathogenicity and resistance

A similar problem happens with the identification and classification of microorganisms with certain pathogenic features. MALDITOF MS has been used recently to identify an *E. coli* clone, ST 131, belonging to the phylogroup B2, and which has been partially responsible for the widespread of ESBLproducing *E. coli* in the last decade [43]. These authors developed specific algorithms that based the belonging to this group on a series of specific peaks in the MS profile, obtaining a high sensitivity and specificity, around or above 90%. However, the results of this study raise two problems. On the one hand, as to the validity of the method to detect this particular clone, it will be necessary to increase the number of isolates analyzed (the study analyzed 105 isolates from both this and other sequence types) to check its sensitivity, specificity and stability over time. On the other hand, this method has been validated for one specific clone belonging to one specific species. More universal algorithms are needed allowing clonality studies and hierarchical clustering in groups of microorganisms belonging to different species with minimal technical changes, if we want this technology to be operational in this field.

The same authors apply a very similar strategy [44] to detect the belonging of *Pseudomonas aeruginosa* isolates to highrisk clones. They identified a series of peaks or combinations of peaks characteristic of each clone, and used these features to assign untyped isolates to specific clones. As in the case of *E. coli*, this strategy is quite reliable for some clones, while for other clones it has a low positive predictive value, less than 60%, although a high negative predictive value. This strategy was also applied to the study of an *E. coli* O104:H4 outbreak, basing the allocation to this serotype on the presence of two characteristic peaks, with good results [45].

Similar strategies have been proposed to recognize microorganisms involved in outbreaks through the detection of characteristic peaks. Some authors have developed a rapid method that allows detecting isolates carrying a carbapenemases-encoding plasmid through the detection of a specific peak [46,47]. A similar approach has been developed [48] for identifying ESBL-producer *E. coli* ST 131 and ST 405.

However, this kind of approaches is barely applicable in epidemiology at general level at this time. The process for detecting and identifying specific peaks associated to specific features (presence of a plasmid, belonging to a specific subtype, etc.), assuming they exist, is slow and complex, and anyway probably useful only for a particular outbreak, having a much lower epidemiological usefulness at a general level. It should be noted that, in the case of plasmids, they are genetic elements undergoing an extremely active exchange of information, in which the information input/output can modify the characteristics of the encoded proteins and therefore of the detected peaks. This approach should be therefore considered useful for specific epidemic situations, but probably limited in time.

Moreover, in studies involving microorganisms with a high number of serotypes [48] it may be difficult to guarantee that an isolate carrying one specific peak belongs to a specific serotype. Probably is more feasible the opposite, i.e., the absence of a specific peak might discard the belonging to a specific genotype.

Anyway, this technology seems to work better when it comes to detect the belonging to specific groups, which some software as ClinProTools® can learn to recognize based on the presence of specific peaks. However, clustering and internal hierarchy of groups of microorganisms of unknown origin seems to be less reliable.

This is a field with a great potential for MALDITOF MS, as it might help to establish hierarchical clustering of great epidemiological interest quickly and with virtually no additional cost added to the microorganism identification. However, there is still a long way to elucidate the real possibilities of MALDI-TOF MS in this field and, if necessary, until the establishment of general and contrasted enough protocols.

Competing Interest

The authors declare to have no competing interests concerning the content of this paper.

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