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Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology

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Abstract

Until recently, microbial identification in clinical diagnostic laboratories has mainly relied on conventional phenotypic and gene sequencing identification techniques. The development of Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS) devices has revolutionized the routine identification of microorganisms in clinical microbiology laboratories by introducing an easy, rapid, high throughput, low-cost and efficient identification technique. This technology has been adapted to the constraint of clinical diagnostic laboratories and has the potential to replace and/or complement conventional identification techniques for both bacterial and fungal strains. Using standardized procedures, the resolution of MALDI-TOF MS allows accurate identification at the species level of most Gram-positive and Gram-negative bacterial strains with the exception of a few difficult strains that require more attention and further development of the

and much quicker than conventional techniques. Recent studies have shown that MALDI-TOF MS has also the potential to accurately identify filamentous fungi and dermatophytes, providing that specific standardized procedures are established for these microorganisms. Moreover, MALDI-TOF MS has been used successfully for microbial typing and identification at the subspecies level, demonstrating that this technology is a potential efficient tool for epidemiological studies and for taxonomical classification.

Introduction

The Matrix-Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF MS) is a rapid, accurate and cost-effective method of microbial characterization and identification. This technology generates characteristic mass spectral fingerprints, that are unique signatures for each microorganism and are thus ideal for an accurate microbial identification at the genus and species levels, and has a potential to be used for strain typing and identification.

Mass spectrometry has been used for several decades in chemistry but it was in 1975 that Anhalt and Fenselau (Anhalt & Fenselau, 1975) proposed for the first time that bacterial characterization could be achieved using this technique. They noticed that unique mass spectra were produced from bacterial extracts of different genera and of different species. In the 1980s, the development of desorption/ionization techniques such as plasma desorption (PD), laser desorption (LD) and fast atom bombardment (FAB) allowed the generation of molecular biomarker ions from microorganisms leading to bacterial profiling (Heller, *et al.*, 1987, Platt, *et al.*, 1988). In early experiments, only biomarker molecules of low mass molecular masses such as bacterial lipids were analysed (Shah & Collins, 1980, Heller, *et al.*, 1988) since the processes used for the ionization of biomolecules were too energy rich to avoid unpredictable analyte decomposition. The evolution of soft ionization techniques such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) developed in the late 1980s (Tanaka & Fenn, 2002) made possible analysis by mass spectrometry of large biomolecules such as intact proteins. Several groups (Cain, *et al.*, 1994, Girault, *et al.*, 1996, Liang, *et al.*, 1996) demonstrated that MALDI-TOF could be used to produce protein profiles following cellular extraction and purification. However, Holland *et*

al. (Holland*, et al.*, 1996) reported for the first time in 1996 that MALDI-TOF spectral fingerprints could be obtained from whole bacterial cells without pre-treatment before the MS analysis. This approach was then used to identify bacteria at the genus and species levels by multiple research teams (Claydon*, et al.*, 1996, Krishnamurthy & Ross, 1996, Haag*, et al.*, 1998, Pribil & Fenselau, 2005, Pignone*, et al.*, 2006, Vargha*, et al.*, 2006). Since the late 1990s, the success of this technique for the rapid identification of bacteria but also fungi and yeast was demonstrated by an impressive exponential increase in the number of publications concerning MALDI-TOF identification of microorganisms.

MALDI-TOF MS has been used to characterize a wide variety of microorganisms including bacteria, fungi, and viruses (Giebel*, et al.*, 2010). The capability of MALDI-TOF to rapidly characterize microorganisms favours its potential applications in multiple areas including medical diagnostics, biodefense, environmental monitoring, and food quality control. MALDI-TOF MS is suitable for high-throughput and rapid microbial identification at low costs and is an alternative for conventional laboratory biochemical and molecular identification systems.

In this review, we will first present the technical background of the MALDI-TOF MS method including sample preparation, mass spectrometry, bioinformatics analysis of fingerprint spectra and their comparison with databases for microorganism identification. We will then present the application of MALDI-TOF MS microbial identification from bacteria and fungi isolates and directly from clinical samples. We will then address the important issues of quality control, maintenance, time-to results and cost effectiveness. Finally, we will discuss the use of MALDI-TOF MS for additional applications such as microbial taxonomy, typing and identification of virulence factors (Bizzini & Greub, 2010, Murray, 2010).

Technique

A mass spectrometer is composed of three functional units, (1) an ion source to ionize and transfer sample molecules ions into a gas phase, (2) a mass analyser that separate ions according to their mass-to-charge ratio (m/z), and (3) a detection device to monitor separated ions. Several ionization methods have been developed including PD, FAB, chemical ionization (CI), atmospheric pressure chemical ionization (APCI), electrospray (ESI), LD, and matrix-assisted laser desorption/ionisation (MALDI). The method of ionization is determined according to the nature of the sample and the goal of the MS analysis, but ESI and MALDI are soft ionization techniques that allow ionization and vaporization of large non-volatile biomolecules such as intact proteins (Emonet*, et al.*, 2010). In contrary to ESI, MALDI generates mostly singly charged ions and thus MALDI derived spectra may include larger numbers of proteins. Laser desorption has been successfully coupled to several kinds of mass analysers to characterize microorganisms such as time of flight (TOF) (Lay, 2001), Fourier transform ion cyclotron resonance (Ho & Fenselau, 1998), quadrupole-TOF (She*, et al.*, 2001) and quadrupole ion trap (Meetani*, et al.*, 2007). The various operative modes of the different mass analysers confer strengths and weaknesses in their performance characterized by mass accuracy, resolution, mass range, sensitivity, scan speed and cost (Table S1) (Jonsson, 2001, Aebersold & Mann, 2003, Domon & Aebersold, 2006, Graham*, et al.*, 2007). The performance of mass analysers can be improved by combining and/or summing the advantages of one sort of analyser (tandem MS) or of different analysers (hybrid MS) through the development of multistage instruments such as hybrid quadrupole time of flight (Q-Q-TOF), tandem time of flight (TOF-TOF) and triple quadrupole (Domon & Aebersold, 2006). Overall, the required performance expected form a mass analyser depends on the type of sample to be analysed (complex/simple mixtures, proteins, peptides, lipids, polysaccharides)

and the ultimate goal of the analysis (quantification, protein identification, microorganism identification, biotyping).

Time of flight mass analysers (Cotter, 1997) have been used for intact microorganisms detection for many years (Heller, *et al.*, 1987) because they are suited for interfacing with pulse laser ionization and offer the possibility of rapid analysis and miniaturization.

In MALDI analysis, samples are prepared by mixing the samples with a matrix which results in the crystallisation of the sample within the matrix. The matrix is composed of small acid molecules that have a strong optical absorption in the range of the laser wavelength used. The matrix composition varies according to the biomolecule to be analysed and the type of laser used (Fenselau & Demirev, 2001). The most frequently used matrices are 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), ferulic acid (FA) and 2,4-hydroxy-phenyl benzoic acid (HPBA). Ferulic acid, sinapinic acid and CHCA have been shown to be effective for the detection of proteins biomarkers (Fenselau & Demirev, 2001, Vaidyanathan, *et al.*, 2002, Williams, *et al.*, 2003) whereas DHB appears to be the best choice for the detection of glycopeptides and glycoproteins (Giebel, *et al.*, 2010). The size and the intensities of the peaks of the detected molecules are dependent on the matrix selected for the experiment. DHB and CHCA are usually optimal for the detection of lower mass ions (Hathout, *et al.*, 2000, Williams, *et al.*, 2003, Ruelle, *et al.*, 2004) with a detection up to 10kDa when the proper solvent is used. Both SA and FA have been shown to be better for the detection of higher mass ions (above 15kDa) (Madonna, *et al.*, 2000, Conway, *et al.*, 2001, Ruelle, *et al.*, 2004, Vargha, *et al.*, 2006) but provide a lower sensitivity than CHCA (Wang, *et al.*, 1998, Ruelle, *et al.*, 2004).

Intact microorganisms can be directly processed to MALDI-TOF without pre-treatment because most vegetative bacteria are lysed following exposure to water, organic solvent

and/or strong acid in the MALDI matrix. When resistant microrganims such as some viruses, bacterial spores and yeast cells have to be analysed by MALDI, strong organic acids and/or alcohols are usually added in pre-treatment steps. Similarly, for some bacterial species (such as the *Actinomyces*), specific pre-treatment or protein extraction procedures may be useful (Bizzini*, et al.*, 2011).

The marked differences in bioanalytes fingerprints observed with the different matrix demonstrate that similar standardized preanalytical and analytical procedures than those performed to establish a database must be followed to ensure accurate identification. This implies that any modification of the procedure (i) should conformed to the manufacturer's recommendations, (ii) should be analysed to demonstrate that protein profiles remained consistent with database fingerprints or (iii) should use a new database created with the modified protocol.

In practice, a microbial sample is mixed with a matrix on a conductive metal plate. The mixture can be deposited on the metal support or alternatively the microbial sample is deposited and dried out on the support before the addition of the matrix. After the crystallisation of the matrix and compound, the target on the metal plate is introduced in the mass spectrometer where it is bombarded with brief laser pulses from usually a nitrogen laser (Figure 1). The matrix absorbs energy from the laser leading to the desorption of the analytes that are then vaporised and ionized in the gas phase. This matrix-assisted desorption and ionization of the analytes leads to the formation of predominantly singly charged sample ions. The desorbed and ionized molecules are first accelerated through an electrostatic field and are then ejected through a metal flight tube that is subjected to a vacuum until they reach a detector, with smaller ions travelling faster than larger ions. The time of flight required to reach the detector is dependent on the mass (m) and charge (z) of the bioanalyte and is proportional to the square root of m/z. Thus, bioanalytes with different m/z that composed a

complex sample are separated according to their time of flight (TOF) and create a mass spectrum that is characterized by both the m/z and the intensity of the ions, which is the number of ions of a particular m/z that struck the detector. The results of a spectral signature is composed of spikes ranging usually from 1000 to 20'000 m/z. Usually, MALDI produces single charged (z=1) ions and thus the m/z of an analyte corresponds to the value of its mass.

Data analysis

Biomarkers

Several research groups have demonstrated that biomolecules desorbed from whole unfractionated cells and detected above 4 kDa are intact proteins (Arnold*, et al.*, 1999, Dai*, et al.*, 1999, Holland*, et al.*, 1999, Ryzhov & Fenselau, 2001). Most of the biomarkers detected in MALDI-TOF spectra of intact bacterial cells have a molecular mass below 15kDa. A thorough characterization of MALDI biomarkers performed on intact *Escherichia coli* cells have demonstrated that the MALDI detected biomolecules corresponds to proteins from the inside of bacterial cell that are abundant, basic and of medium hydrophobicity (Ryzhov & Fenselau, 2001). Among these MALDI-detected proteins, about half matched ribosomal proteins that are abundant and very basic (Arnold*, et al.*, 1999, Ryzhov & Fenselau, 2001), which is a biochemical trait favourable for efficient ionization during the MALDI process (Krause*, et al.*, 1999). Thus, the abundance (more than 20% of total cell proteins) and the basic nature of ribosomal proteins explain why the majority of the peaks detected in a MALDI-TOF spectrum correspond to ribosomal proteins. In addition, the lysis of bacterial cells in organic solvents and in acidic conditions favouring the extraction of ribosomal proteins combined with the utilisation of a specific matrix allowed the development of a method leading to the ionization of mainly ribosomal proteins (Suh & Limbach, 2004).

In a study performed by Ryzhov *et al*. on *E. coli* to characterize the nature of the proteins favoured by MALDI, several additional group of proteins than ribosomal proteins were

identified (Ryzhov & Fenselau, 2001). These also included abundant nucleic acid-binding protein, such as *E. coli* DNA-binding protein HU α- and β-subunits (DbhA and DbhB, respectively) and cold-shock proteins, such as cold-shock proteins A, C and E (CspA, CspC, CspE). Similar to ribosomal proteins, these protein families are highly abundant, basic and of medium hydrophobicity. Holland *et al.* potentially identified the acid-resistant precursor proteins HdeA and HdeB observed in the MALDI analysis of both intact *E. coli* and *Shigella flexneri* (Holland, *et al.*, 1999). The ion at m/z 7643 in the spectra from *Pseudomonas aeruginosa* was mapped to the cold-shock protein CspA and similarly the ion at m/z 7684 observed in *P. putida* was identified as the cold-acclimation protein CapB (Fenselau & Demirev, 2001). Sun *et al.* have selected in the MALDI-TOF spectra of *Lactobacillus plantarum* 34 reliable biomarkers including 31 ribosomal subunit proteins and 3 ribosome associated proteins identified as a small heat shock protein, a methylase and the DNA-binding protein II (Sun, *et al.*, 2006). A comprehensive study was published by Dieckmann *et al.* where protein identities were assigned to biomarker peaks obtained by whole-cell MALDI-TOF MS of salmonellae (Dieckmann, *et al.*, 2008). Most of the proteins identified in this study were abundant cytosolic proteins that were highly basic including in particular ribosomal proteins, proteins involved in DNA or RNA binding, and other abundant proteins, most of which having a high isoelectric point greater than 9. Thus, examples of biomarker peaks assigned to proteins characterized by a high isoelectric point included not only ribosomal proteins but also many other proteins such as cold shock-like protein CspH, translation initiation factor IF-1 (pI 9.23), DNA binding protein HU α- and β- (pI 9.69 and 9.57), the ribosome modulation factor (pI 10.56), and integration host factors A and B (both pI 9.34). Lower pI values proteins detected in this study were in general very abundant proteins including the nucleoid-associated protein H-NS (pI 5.32), the RNA chaperone CspE (pI 8.08), glutaredoxin-1 (pI 5.63) and the phosphocarrier protein HPr (pI 5.6).

Thus, most of MALDI-TOF spectra are composed of very conserved proteins with house-keeping functions affected to a minimal extent by environmental conditions and thus considered to be optimal for routine identification of bacteria.

Biomarkers Reproducibility

The reproducibility of MALDI-TOF spectrum of whole bacterial sample is problematic since large variations can be seen in spectra of the same bacterial species obtained in different conditions. Many experimental parameters can have an important effect on the observed mass spectra (Wang*, et al.*, 1998). The reproducibility is dependent on the MALDI-TOF instrument, the matrix used, the age of the microorganism, the sample:matrix ratio, the sample concentration, the culture medium and growth conditions (Valentine*, et al.*, 2005). However, several studies have shown that a subset of peaks from genetically identical bacteria was conserved in spectra obtained in different experimental conditions (Wang*, et al.*, 1998, Welham*, et al.*, 1998). These conserved peaks, among which ribosomal proteins are well represented, explain the feasibility to use MALDI-TOF for bacterial identification even without the standardization of experimental conditions. These results also suggest that selected specific conserved biomarker proteins could be used for bacterial identification irrespective of changes in other biomarkers. However, to optimize the reproducibility, a standardization of sample preparation (e.g. choice of matrix, concentrations, solvent and crystallization conditions) has to be established by diagnostic laboratories.

Intra-laboratory reproducibility

Only a few studies have reported investigations on intra-laboratory reproducibility. In two studies that were focusing only on the presence or absence of particular peaks but not on peak intensities, the level or reproducibility obtained was equal to 75% or higher using the same mass spectrometer and similar sample preparation techniques (Saenz*, et al.*, 1999, Walker*, et al.*, 2002). The reproducibility of MALDI-TOF MS fingerprints was high even when mass

spectra were obtained from bacterial cultures propagated in the same standard culture conditions during weeks and even months (Bernardo*, et al.*, 2002).

In our laboratory, we observed that the reproducibility is mainly dependent on the quality of the deposit and that the extraction step is not associated with significant supplementary variability. Thus, when considering the higher peaks (≥200 intensity units) obtained with *E. coli* strain ATCC 25922 in 10 independent analyses, 66 peaks were present in all 10 replicates performed with proteins obtained from the same extraction whereas 78 peaks were present in the 10 spectra obtained from 10 independent extractions (Figure 2A). Similar results were obtained with *S. aureus* strain ATCC 25923 (Figure 2B).

Inter-laboratory reproducibility

The variation of inter-laboratory reproducibility is not surprisingly much higher since small variation in sample preparation and analysis may significantly affect mass fingerprints. Unfortunately, only a few attempts have been made to compare results from different laboratories on the same organism. In addition, inter-laboratory studies based on different comparative settings have been performed and gave very different results. Studies comparing mass spectra of identical microorganisms obtained in different locations but with the same experimental protocols and instruments hardware and software have shown promising results in term of inter-laboratory reproducibility. For instance, two studies using different bacterial species have demonstrated that more than 60% of the peaks observed in mass spectra were similar in separate laboratories (Wang*, et al.*, 1998, Walker*, et al.*, 2002). However, a poor inter-laboratory reproducibility of MALDI-TOF MS of intact microorganisms was demonstrated in a study where three independent laboratories using three different commercial instruments performed a MALDI-TOF MS analysis of identical aliquots of *E. coli* culture prepared and analysed in the same experimental conditions (Wunschel*, et al.*, 2005). In this study, only 25% of the biomarkers were found in common by all three laboratories and

more than 50% of the peaks were detected in spectra from only one of the three laboratory. Of importance, when the mass spectra collected from the instrument of one of the laboratory was analysed in the other two laboratories, 70% of the mass fingerprints could be identified correctly as *E. coli*. This finding underlines the importance of the instrument in the establishment of bacterial fingerprint databases.

Application for microorganism identifications

Two general MALDI-TOF MS methods have been proposed to characterize microorganisms: (1) mass spectra comparison with fingerprints database and (2) matching of biomarker masses to a proteome database. In the first approach, generated unique spectra of intact cells are compared with previously collected fingerprint libraries that are commercially available. This solution is rapid, simple and is easily adaptable for routine use in diagnostic laboratories. This approach is convenient to develop specific databases constituted of unique and conserved peaks that can be used for species and subspecies identification, independently of the culture conditions used to grow the microorganism (Carbonnelle*, et al.*, 2007). In the second approach, the biomarker masses associated with an unknown microorganism are identified by matching protein molecular masses in the spectrum with protein molecular masses predicted from sequenced genomes (Demirev*, et al.*, 1999). This method is based on the observation that the majority of observed biomolecules above 4000 m/z in MALDI-TOF spectra of whole cell extracts are proteins. An available algorithm predicts protein masses *in silico* from the genomes and seeks matches with experimentally derived masses (Pineda*, et al.*, 2000). However, this application is limited to microorganisms whose genomes are sequenced and further development in strategies for organization of the proteome database is required. The advantage of such a bioinformatics-based approach compared to bacterial fingerprinting is that the identification tolerates variations in the protein profiles and thus differences in culture growth and sample treatment conditions.

Bacterial identification

The identification of microorganisms in clinical diagnostic microbiology laboratories is nowadays mainly performed by analysis of biochemical reactions and phenotypic characteristics, such as growth on different media, colony morphology and Gram staining. When combined, these routine laboratory techniques ensure an accurate identification of most microorganisms but are costly, require time and need in some cases well-trained technician for correct interpretation. One of the major advantages of using MALDI-TOF technology for bacterial identification is the time-to-result, which is reduced from 24-48h to less than an hour. In addition, MALDI-TOF allowed accurate bacterial identification of a large variety of bacteria, that only exhibit few phenotypic traits and that were identified by 16S rRNA gene sequencing prior to the MALDI-TOF era (Bizzini*, et al.*, 2011).

Routine identification

Several approaches are commonly used in routine diagnostic laboratories. One typical approach is to pick bacterial isolates colonies freshly grown on defined agar medium with a sterile tip and to smear a thin film onto a ground steel MALDI target plate. The microbial film is then overlaid with a MALDI matrix selected as recommended by the MALDI-TOF manufacturers, typically 1.5 µl CHCA in 50% acetonitrile/2.5% trifluoroacetic acid for the Bruker instrument and 0.5 µl of 20mg DHB dissolved in 1ml water-ethanol-acetonitrile [1:1:1] mix for the Shimadzu instrument. In our laboratory, to increase identification yield, we routinely add formic acid on smeared micro-organisms before adding the matrix. The sample-matrix mixture is dried at room temperature and then introduced in the MALDI-TOF instrument for data acquisition. The data are processed by the associated softwares (see above) and the spectra are compared to reference libraries for bacterial identification. A protein extraction step using ethanol-acetonitrile is performed when direct application procedure failed.

Several studies have analysed the bacterial identification efficiency of these two instruments (Bruker and Shimadzu) with their respective software and databases (Table 1). We will present here the results of (1) two intra-laboratory studies using the Bruker system (Seng*, et al.*, 2009, van Veen*, et al.*, 2010), (2) an intra-laboratory study evaluating the two instruments using the same microbial samples (Cherkaoui*, et al.*, 2010), and (3) a large international interlaboratory study performed with the Bruker instrument (Mellmann*, et al.*, 2009).

Using the Bruker system, 327 clinical isolates previously identified by conventional techniques were analysed by MALDI-TOF MS by Van Veen *et al*. The authors observed a 95.1% correct identification at the genus level and a 85.6% at the species level (van Veen*, et al.*, 2010). The same group performed a prospective validation study on 980 clinical isolates of bacteria and yeast that showed a 92.2% correct identification by MALDI-TOF, a performance significantly better than the 83.1% identification obtained with conventional biochemical systems. Correct species identification by MADLI-TOF MS was observed in 97.7% of *Enterobacteriaceae*, 92% of nonfermentative Gram-negative bacteria, 94.3% of staphylococci, 84.8% of streptococci, 84% of bacteria of the HACCEK group (*Haemophilus*, *Actinobacillus*, *Cardiobacterium*, *Capnocytophaga*, *Eikenella* and *Kingella*) and 85.2% of yeasts. In this work, misidentification was clearly associated with insufficient spectra from suitable reference strains in the reference spectra database.

Seng *et al*. have conducted a prospective routine MALDI-TOF MS identification analysis with the Bruker system on 1660 bacterial isolates in parallel with conventional phenotypic bacterial identification (Seng*, et al.*, 2009). Discrepancies were resolved by 16S rRNA and *rpoB* gene sequencing. They have shown that 95.4% of the isolates were correctly identified by MALDI-TOF MS among which 84.1% at the species level and 11.3% at the genus level only. Absence of identification (2.8% of isolates) and incorrect identification (1.7% of isolates) were mainly due to improper database entries. They have estimated that the MALDI-

TOF identification required an average time of 6 minutes for an estimated 70-80 % reduced cost compared to conventional methods of identification.

Cherkaoui *et al.* have evaluated the two main MALDI-TOF MS systems, Bruker and Shimatzu, in a comparative study with 720 bacterial isolates under routine clinical laboratory conditions (Cherkaoui*, et al.*, 2010). The isolates were analysed in parallel on both devices according to the manufacturers' default recommendations. The MALDI-TOF MS results were compared with conventional biochemical identification tests and discordant results were resolved with 16S rRNA gene sequencing. The Bruker MS system gave high-confidence identification for 680 of 720 isolates (94.4%) whereas the Shimadzu MS showed a high-confidence identification for 639 isolates (88.8%). These results showed also that only 6/680 (0.9%) of the Bruker and 3/639 (0.5%) of the Shimadzu identifications gave an incorrect high-confidence identification at the species level. All the high-confidence MS identifications were accurate at the genus level. In addition, the Bruker MS system has identified 9 (69%) and the Shimadzu system 5 (38%) of 13 isolates that were not identified by conventional phenotyping methods.

Interlaboratory species identification was assessed in a large international multicenter study (Mellmann*, et al.*, 2009) using the Bruker system. In this study, eight participating laboratories received 60 blind-coded samples for MALDI-TOF MS species identification and 98.75% were correctly identified at the species level. Six of the eight laboratories identified all 60 samples correctly. Out of a total of 480 samples, 6 samples were misidentified and one sample did not give any valid result due to low signal intensity. Thus, in contrast to other studies (Wang*, et al.*, 1998), the utilization of a commercial system developed for routine use provided high reliability for bacterial identification.

Even though excellent results have been shown for bacterial identification by MALDI-TOF MS, both the diagnostic yield (identification at the genus, species or strain level) and the accuracy of identification depends on the taxonomy and in the quality of the databases. As shown by many studies, most of the bacterial groups including *Enterobacteriaceae*, nonfermentative Gram-negative bacteria, staphylococci and haemolytic streptococci were correctly identified as the species level (Seng*, et al.*, 2009, Cherkaoui*, et al.*, 2010, van Veen*, et al.*, 2010). Regarding staphylococci, the MALDI-TOF MS has brought up an important advantage by allowing rapid and simplified identification of both *S. aureus* and of some species belonging to coagulase-negative staphylococci (CoNS). Unlike most commercial identification systems that allow a rapid identification of only *S. aureus*, the MALDI-TOF MS allows a correct identification of various CoNS species (Speers*, et al.*, 1998, Bernardo*, et al.*, 2002, Dupont*, et al.*, 2010). In a comparative study between MALDI-TOF MS and two rapid identification automated systems, BD Phoenix (BD Diagnostic Systems, France) and Vitek-2 (bioMérieux, France), the identification of 234 CoNS belonging to 20 different species showed that the MALDI-TOF performance was significantly better (93.2%) than Phoenix (75.6%) and Vitek-2 (75.2%) (Dupont*, et al.*, 2010). Overall, MALDI-TOF appears to be excellent at identifying various staphylococci species as demonstrated by the congruence of 99.3% (444/447) between MALDI-TOF and *rpoB* sequence-based identifications (Spanu*, et al.*, 2011). Since CoNS can cause serious infections and are frequently associated with hospital-acquired infections, the rapid identification at the species level by MALDI-TOF is very useful in distinguishing clinically significant CoNS from contaminant strains (von Eiff*, et al.*, 2002).

Problematic identifications

Most of the problematic identifications encountered in most of the recent studies concerned the viridans streptococci group, the pneumococci and anaerobic bacteria (Table 2). Viridans

streptococci and pneumococci were commonly misidentified mainly due to an incomplete database reference library. In the study by Seng *et al.*, nearly 50% of *S. pneumoniae* isolates were misidentified as *Streptococcus parasanguinis* (a close related species within the mitis group of *Streptococcus* species (Kawamura*, et al.*, 1995)) because the database contained only three *S. pneumoniae* and two *S. parasanguinis* reference spectra (Seng*, et al.*, 2009). The problem was solved by adding additional *S. pneumoniae* isolates reference spectra to the database which clearly indicates that the database need to be updated with multiple spectra of well-characterized streptococcal species. Until now, the identification of *Streptococcus* spp. remains a problem for MALDI-TOF MS identification especially for closely related species such as *S. pneumoniae*, *S. mitis* and *S. parasanguinis*. In the case of pneumococci, the use of MALDI-TOF MS for identification is further impaired by the weak extraction yield caused by the presence of a capsule. Thus, identification of *S. pneumoniae* should not solely rely on MALDI-TOF since false identification can results in important clinical outcomes. In the study of Cherkaoui *et al*., the diagnostic yield for streptococcal species and for Gram-negative anaerobes was less than 50% with an accuracy of high-confidence species identification of only 57.1% for streptococci with the Bruker system and of 71.4% with the Shimadzu MS system (Cherkaoui*, et al.*, 2010).

To date, only a few studies have analysed the usefulness of MALDI-TOF for routine identification of anaerobic bacteria. Seng *et al*. showed that an improved database is required since 50% of the total isolates (46) showing no identification by MALDI-TOF MS were anaerobic bacteria including *Fusobacterium nucleatum* and non-*Clostridium* anaerobes that had no reference in the Bruker database (Seng*, et al.*, 2009). In contrary, for anaerobic species (e.g several *Bacteroides* species) with sufficient spectra in the database, the identification by MALDI-TOF MS was better compared to conventional biochemical methods. These fastidious organisms are poorly identified using phenotypic methods with a

lack of specificity and ambiguous or false identification. There is thus an important need to improve the database entries with additional anaerobes isolates (Bessede*, et al.*, 2011).

Similar to other studies, Blondiaux *et al*. have demonstrated the difficulties to identify viridans streptococci, pneumococci as well as HACCEK bacteria but also *Shigella* and several strictly aerobic bacteria (*Aeromonas* spp., *Achromobacter* spp., *Alcaligenes* spp.) (Blondiaux*, et al.*, 2010). In this study, the mass spectra of six *Shigella* isolates were similar to several *E. coli* strains present in the Biotyper database. In another study, the misidentification of all *Shigella sonnei* isolates with *E. coli* was also documented (Seng*, et al.*, 2009).

Using 43 selected *Mycobacteria* strains, a mycobacterial database could be engineered comprising species-specific spectral profiles allowing identification of 44 species at the species level and of 9 strains of the *M. abscessus* complex and the *M. tuberculosis* complex at the mycobacterial clade level (Lotz*, et al.*, 2010). Under the preanalytical and analytical conditions used in this study, subspecies of the *M. abscessus* complex (*M. abscessus*, *M. massiliense* and *M. bolletti*) and the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. microti* and *M. africanum*) produced indistinguishable mass profiles due to their high degree of genetic similarity. Using this microbial database, 311 strains grown on solid medium were analysed by MALDI-TOF MS allowing a 97% correct identification, 67% at the species level and 30% at the complex level respectively. No misidentification was observed. When bacteria were grown on liquid media, correct identification was reduced to 77%, likely due to a reduced number of bacterial load or to potential interference with components of the liquid media. Interestingly, the authors observed than an increase in number of replicates (up to 5) did correlate with an increase probability of good identification, especially for slow-growing mycobacteria. Overall, several studies demonstrate that MALDI-TOF MS provides high reproducibility and specificity for mycobacterial identification and represents an alternative to

other time consuming and fastidious conventional mycobacterial identification methods (Hettick*, et al.*, 2004, Lefmann*, et al.*, 2004, Pignone*, et al.*, 2006, Lotz*, et al.*, 2010).

In our study analysing the performance of MALDI-TOF MS for the identification of 1371 bacterial isolates routinely isolated in clinical microbiology laboratories and characterized by conventional methods, 1278 (93.2%) bacterial isolates were identified at the species level, 73 (5.3%) were only identified at the genus level and 20 (1.5%) gave no reliable identification (Bizzini*, et al.*, 2010). Among the 1278 isolates identified at the species level, 63 (4.9%) were misidentified. The majority of discordant results (42/63) were explained by discordances due to the MALDI-TOF database, 14 were due to poor discrimination of the spectra of closely related species such as *Shigella* spp. and *E.coli*, and 7 were caused by errors in the initial conventional phenotypic and biochemical identifications.

Many of the bacterial identification that can be done only at the genus level are due to incomplete reference spectra covering many different isolates or species from a given genus. For instance, only 1 reference spectrum of *P. acnes* (strain DSM 1897) or *Bacillus cereus* are included in the Bruker database which is totally insufficient to cover the true diversity of these bacteria and thus to identify accurately these microorganisms (Bizzini*, et al.*, 2010) . In addition, mislabelling of bacterial species in the database can cause misidentification by MALDI-TOF MS. In the study by Seng *et al.*, 7 *S. maltophilia* isolates were incorrectly identified as *P. hibiscicola* which is an invalid name for a nonfermenting Gram-negative rod that was demonstrated to be *S. maltophilia* (Seng*, et al.*, 2009).

These studies show that a complete and representative database is an essential requirement for accurate identification of isolates by MALDI-TOF MS. A frequent update of the reference library database with spectra of appropriate poorly represented reference strains by the

manufacturers but also by routine diagnostic laboratories can significantly impact the MALDI-TOF identification performance.

Impact of Sample preparation on MS identification

Sample preparation has not been shown to be a major issue for problematic identifications. Samples and matrix prepared according to manufacturer's instructions usually guarantee high quality spectral fingerprints required for efficient MALDI-TOF identifications. In most cases, the composition of an appropriate matrix containing strong solvent ensures efficient bacterial lysis required for MALDI-TOF analysis. When invalid results are initially obtained by MALDI-TOF MS following analysis of intact bacteria directly deposited on MALDI target plate, a step of bacterial protein extraction with acid-containing sample solvents improving cell lysis solve the problem in most cases. Bizzini *et al*. have shown that a formic acid-acetonitrile extraction step was required to get a valid MALDI-TOF MS identification for 25.6 % of the 1278 valid isolates (Bizzini*, et al.*, 2010). The yield of valid score from direct application was almost the same, about 75%, for both Gram-positive and Gram-negative bacteria. For instance, the yields of valid scores without extraction step were equal to 79% for *S. aureus*, 82% for *Enterococcus faecalis*, 92% for *Pseudomonas aeruginosa*, 74% for *E. coli*, 58% for *Klebsiella pneumoniae* and 58% for *Staphylococcus epidermidis*. However, the authors concluded that protein extraction prior to MALDI-TOF analysis should be performed only in particular cases known to be problematic such as colonies isolated from urine culture devices and/or MacConkey agar which contains crystal violet, a possible interfering substance affecting mass peak signals. Indeed, the higher identification rate obtained after an extraction step largely compensates the longer hand-on-time associated with an extraction procedure.

This was also demonstrated by a study performed by Liu *et al.* that have developed a universal sample preparation for characterization of bacteria by MALDI-TOF MS (Liu*, et al.*, 2007). The protocol, consisting of a pretreatment of bacteria with acidic sample solvents and

mixing with CHCA or CMBT matrix, could be used to analyse both Gram-positive bacteria, including spore-producing *B. anthracis* and non-spore-producing *S. aureus*, and Gram-negative bacteria such as *Y. pestis*, *E. coli*, and *B. cepacia* that are characterized by high extracellular-polysaccharide contents.

However, for some microorganisms, insufficient cell lysis and/or low quantity of sample material can be problematic for efficient MALDI-TOF identification and require the use of alternative identification approaches such as 16S rRNA gene sequencing. A study by Bizzini *et al*. focusing on the identification of 410 clinical isolates that could not be identified with conventional laboratory methods showed that 133/410 (32.4%) isolates could not be either reliably identified with MALDI-TOF (Bizzini*, et al.*, 2011). The failure to obtain a reliable identification was due to the absence of reference spectra in the BioTyper database for 58% (78/133) of the isolates and to poor protein spectral signals for 41.4% (55/133) of the isolates. The authors supposed that the poor spectrum quality observed could be due to either the difficulty to lyse the cell wall of some bacteria such as Gram-positive bacilli and/or to fastidious growth of some isolate, which yielded only small amount of available sample material. Difficult to identify microorganisms belonging to the genus *Actinomyces*, *Gemella*, *Nocardia* and *Streptomyces* could be observed in this study.

The rate of successful identification is directly linked to the amount of microorganism available. We have observed that the rate of correct identification is especially poor when the subculture has been incubated on a plate for less than 4 hours (Figure 3). Thus, when we tried to identify various bacterial species isolated from positive blood cultures and sub-cultured on agar, we observed that only 30% of bacteria could be successfully identified after 2 hours of incubation (Figure 3C). This identification rate was significantly lower for Gram-positive cocci, remaining below 60% after 6 hours. This is likely due to the amount of bacteria available after short incubation period (Figure 3B). Indeed, using ten-fold dilutions of *E. coli*

strain ATCC 25922 and *S. aureus* strain ATCC 25923, we could show that as much as 10^6 bacteria/well were necessary to consistently obtain a spectrum (Figure 3A) ensuring a score above 2 and that when the amount of bacteria was ranging between 10^2 to 10^4 bacteria/well, the score was always below 1.7 (data not shown). Lower amount of bacteria may however be identified with a different identification algorithm. Hsieh *et al.* have demonstrated that as few as 5 x 10^3 cells from a pure strain can be identified by MALDI-TOF MS using particular analysis approaches based on selected markers (Hierarchical Clustering Analysis and direct classification model construction) (Hsieh, *et al.*, 2008). Interestingly, using the classification model analysis, the authors showed that successful MALDI-TOF MS identification can also be achieved from a bacterial species mixture consisted of as less as 3 x 10^4 cells. This study opens new perspectives for the direct identification of low abundant bacteria located in mixed flora without the pre-requirement of bacterial isolation and culturing.

Fungal identification

Fungal identification still largely relies on phenotypic traits. However, a few days are necessary to obtain mature fungi with phyalids (phyalids are conidiogenous cells observed in a type of fungal asexual reproduction leading to the production of conidia). This time delay may be important given the morbidity and mortality of fungal infections, especially common and life threatening among neutropenic patients. Thus, like PCR and sequencing, MALDI-TOF has the potential to provide accurate and objective identification at species level, with the additional advantage of rapidity and reduced costs compared to PCR and sequencing.

MALDI-TOF MS systems for the identification of microorganisms was successfully adapted for the identification of fungi in the past 10 years. In 2000, Welham *et al.* were among the first to perform fungal identification using the MALDI-TOF MS approach (Welham, *et al.*, 2000). Three fungal species, *Penicillium* spp., *Scytalidium dimidiatum* and *Trychophyton*

rubrum, showed distinct spectral fingerprints allowing accurate species distinction. Since then, many studies have demonstrated the usefulness of the MALDI-TOF application for the identification of various fungal groups such as penicillia, aspergilla, *Fusarium*, *Trichoderma* and dermatophytes. However, until now, MALDI-TOF MS is mainly used for the routine identification of yeasts whereas further development has to be accomplished in database libraries and sample preparation protocols in order to implement this identification approach to other group of fungi such as filamentous fungi and dermatophytes.

Yeasts

The databases of the associated softwares (Biotyper and SARAMIS) of the two main MALDI-TOF instruments contains reference spectra of multiple clinical yeast isolates, including several *Candida* spp. and *Cryptococcus neoformans*, which allows the use of MALDI-TOF in routine yeast laboratory identification (Table 1).

Marklein *et al*. evaluated MALDI-TOF MS for the rapid routine identification of clinical yeast isolates and showed that 92.5 % (247/267) of clinical isolates of *Candida*, *Cryptococcus*, *Saccharomyces*, *Trichosporon*, *Geotrichum*, *Pichia*, and *Blastoschizomyces* spp. were accurately identified (Marklein*, et al.*, 2009). In a study performed by Van Veen *et al*., 85% of 61 yeast isolates comprising 12 different species were correctly identified without occurrence of major errors (van Veen*, et al.*, 2010). Bizzini *et al*. achieved a 100% correct identification of 24 yeast isolates belonging to 12 different species (Bizzini*, et al.*, 2010). The suitability of the two commercially available MALDI-TOF MS systems, Bruker and Shimadzu, and their respective associated softwares and databases, Biotyper and Saramis, was tested for rapid species identification of yeasts in a clinical diagnostic approach (Bader*, et al.*, 2010). Both MALDI-TOF MS systems have showed a similar species identification rate of 97.6 % for Bruker/Biotyper and 96.1 % for Shimadzu/Saramis that were comparable to the biochemical tests rate (96.9%). Based on isolates that were contained in the respective

database, no misclassifications were seen with Saramis and fewer misidentifications were reported by the Biotyper compared to classical approaches. Using the Bruker MALDI-TOF system, Stevenson *et al*. have created a spectral database library for 109 reference strains of yeast representing 44 species and 8 genera to evaluate the use of MALDI-TOF MS for the rapid identification of yeast species (Stevenson*, et al.*, 2010). This library was challenged with 197 clinical isolates. Three isolates gave no spectral score since no reference spectrum were included in the database library. Of the remaining 194 clinical isolates, 192 (99.0%) were correctly identified at the species level and two organisms gave consistently low spectral scores that could not be identified. In summary, the use of MALDI-TOF mass spectrometry for the identification of clinically relevant yeasts is rapid and accurate providing that the database is constructed with a comprehensive collection of accurately identified reference strains.

Filamentous fungi

Chen *et al.* successfully identified several *Penicillium* species directly from intact fungal spores mixed with the MALDI matrix (Chen & Chen, 2005). Hettick *et al.* have achieved 100% correct identifications of 12 *Penicillium* species by bead beating fungal samples resuspended in a acetonitrile/trifluoroacetic acid solvent prior to MALDI-TOF analysis (Hettick*, et al.*, 2008). The observed mass spectra contained abundant peaks in the range 5000-20000 m/z allowing unambiguous discrimination between species. In addition, a biomarker common to all *Penicillium* mass fingerprints was observed at m/z of 13.9 kDa.

Using an extraction method similar to that used for *Penicillium* species, Hettick *et al.* obtained also highly reproducible mass spectral fingerprints for 12 species of *Aspergillus* and 5 strains of *A. flavus* (Hettick, *et al.*, 2008). The 12 species were correctly identified but only a 95% accurate identification was obtained at the strain level. It was also pointed out that *Aspergillus niger* could not be distinguished from *Aspergillus chevalieri*. The authors concluded that the

identification of *Aspergillus* spp. with MALDI-TOF MS would require a comprehensive database of at least 180 species of *Aspergillus*. A study showed that different species of *Aspergillus*, including aflatoxigenic and non-aflatoxigenic spp., could be characterized directly from intact spores (Li*, et al.*, 2000). However, the authors reported certain discrepancies due to the difficulties encountered to discriminate the spectra obtained with some of the analysed species. A database including the reference spectra of 28 clinically relevant *Aspergillus* species was engineered in a recent study by including species-specific fingerprints of both young and mature colonies of reference strains (Alanio*, et al.*, 2010). The performance of the database was tested on 124 clinical and 16 environmental *Aspergillus* isolates resulting in a 98.6% (138/140) correct identification with 100% specificity (0% misidentification). This study has demonstrated that a complete fingerprint database including spectra from both young and mature fungal colonies makes MALDI-TOF a robust method for *Aspergillus* species identification regardless of the maturity of the tested isolates.

The identification of multiple *Fusarium* spp. has also been demonstrated by various studies. In the study by Marinach-Patrice *et al*., 62 strains or isolates belonging to 9 *Fusarium* spp. were subjected to both molecular identification and MALDI-TOF MS analysis (Marinach-Patrice*, et al.*, 2009). Following updating of the BioTyper database with 13 strains of 5 *Fusarium* spp., 57 (92%) strains were correctly identified by MALDI-TOF MS analysis. Only one *Fusarium pseudonygamai* isolate was misidentified and four *Fusarium* isolates were not identified due to absence of reference spectra in the database. MALDI-TOF MS was also used successfully to identify 5 mycotoxin-producing *Fusarium* spp. by direct analysis of spores which yielded highly reproducible MS profiles (Kemptner*, et al.*, 2009).

Dermatophytes

The most important clinical fungal dermatophytes species, *T. rubrum*, *Trychophyton interdigitale*, *Trychophyton tonsurans* and *Arthroderma benhamiae*, originating from skin and

nail were recently identified using the SARAMIS database (Erhard*, et al.*, 2008). Except for one *T. rubrum* strain, a high level of confidence (99.9%) was obtained in this study where sufficient MS spectra were used to produce a super-spectrum for each species.

Problematic identifications

Similar to bacteria, absence of identification or misidentification of fungal species by MALDI-TOF MS analysis are essentially due to absence, mistakes or incomplete reference spectra in the database (Table 2). High quality MS spectra are usually easily obtained with both fungal hyphae and spores following manufacturer's instruction or based on the recommendation of reference studies. For instance, because of their cell wall structure, yeasts need an extraction step to yield a valid score of identification by MALDI-TOF MS. In the study of Bizzini *et al.*, only 4% (1/24) of the valid results were obtained by direct application of the colony on the MALDI plate (Bizzini*, et al.*, 2010) and an extraction method prior to analysis by MALDI-TOF was also shown to be mandatory to obtain appropriate spectra in the study performed by van Veen *et al.* (van Veen, *et al.*, 2010). The spectra of several *Penicillium* spp. obtained from bead beating fungal samples resuspended in an acidic solvent prior to MALDI-TOF analysis was more discriminative (abundant peaks in the range 5000-20000 m/z) than spectra obtained directly from intact spores (range of 2600-7378 m/z) (Chen & Chen, 2005, Hettick*, et al.*, 2008). However, Valentine *et al*. identified *Aspergillus niger*, *Rhizopus oryae*, *Trichoderma reesei* and *Phanerochaete chrysosporium* using either intact spores, hyphae or extracts showing that intact cells, sonicated cells and acid-treated cells yielded similar spectra (Valentine*, et al.*, 2002).

The main problem is that, unfortunately, very few reference spectra are currently included in the database of commercially available MALDI-TOF MS systems. Most of the studies showing that the MALDI-TOF MS identification is a powerful system for the characterization and identification of fungi have built and used their own reference spectra database and have

developed their own sample preparation techniques. There is thus still a lack of standardized extraction protocols regarding filamentous fungi.

In addition, the spectral signal of filamentous fungi may be strongly influenced by the phenotype of the fungus including basidiospore, monokaryon, dikaryon, fruiting body, surface mycelium, strands and substrate mycelium. Moreover, vegetative mycelium grown on agar shows multiple zones that correspond to different ages or developmental stages. These variations may thus influence the spectral reproducibility of the same isolate and a comprehensive database of filamentous fungi should include MS fingerprints of several different developmental forms to guarantee high yields and accuracy of identification as demonstrated by Alanio *et al*. (Alanio*, et al.*, 2010).

Direct identification from samples

Given the accuracy of MALDI-TOF for bacterial identification, this technology might be directly applied to some clinical samples, such as blood, urines, cerebrospinal fluid, pleural fluid, peritoneal liquid and synovial fluid. The major limitation is the amount of bacteria present in the samples and the limit of detection of current MALDI-TOF protocols. To circumvent this difficulty, large volumes are used for blood and urines and an additional enrichment by culture is available for blood (see paragraphs below). Regarding cerebrospinal fluid, Nyvang Hartmeyer *et al.* successfully identified *S. pneumoniae* 30 minutes after receiving a sample supporting the proof-of-concept (Nyvang Hartmeyer*, et al.*, 2010). However, practically, bacterial identification from cerebrospinal fluid, strongly limited by the low bacterial load and the limited volume available, is yet not applicable in routine diagnostic laboratories.

Blood (hemoculture)

Bloodstream infection, septic shock and endocarditis represent severe diseases with important mortality and morbidity. Blood culture represents the best way to establish the etiology of such infections and to guide antimicrobial treatment. This is important since rapid and appropriate antimicrobial therapy is pivotal to reduce poor outcome (Kollef, 2000). Indeed, the fatality rate was 20% for bloodstream infection patients treated with appropriate therapy, and 34% for patients treated with inappropriate therapy (Leibovici*, et al.*, 1998). The rapid notification of the Gram stain result from positive blood culture has also a positive impact for adaptation of antimicrobial regimen (Munson*, et al.*, 2003). Consequently, the precise identification of a microorganism isolated from positive blood culture early after Gram stain notification will likely help clinician to better adapt the antimicrobial therapy. As an example, the impact on the choice of the antibiotic will likely be significant when Gram-positive cocci are identified from blood cultures, since the antibiotic susceptibility of *E. faecium* is clearly different from that of alpha-hemolytic streptococci.

Quantitative blood-cultures have demonstrated that bacterial load during bloodstream infection is very low in adults, often less than 1-10 colony forming unit/ml. In practice, blood samples are inoculated into bottles containing broth media and incubated in automated instruments monitoring CO_2 concentrations released during bacterial growth. At the automated growth detection time, the bacterial load may reach a heavy growth up to 10^6 to 10^8 colony forming units/ml. In our laboratory, *Enterobacteriaceae, Pseudomonas aeruginosa* and aerobic Gram-positive cocci were generally detected when present at 10^7 bacteria/ml. Such bacterial concentration might be adequate to allow accurate bacterial identification using mass spectrometry.

However, the blood culture bottle fluid represent a complex solution with multiple non-bacterial proteins isolated from patient's blood and nutrient growth media. These proteins alter the specific bacterial mass spectrometry profile obtained by MALDI-TOF and have a

detrimental effect on the performance of algorithm used to query the database containing bacterial mass spectrometry profiles.

The preparation of a bacterial pellet from positive blood culture includes a differential centrifugation step to discard blood cells, an erythrocyte lysis step and a subsequent washing step to remove additional non-bacterial components. Application of this protocol allows the identification in less than 1 hour as compared to overnight growth of bacteria required to obtain pure colonies for biochemical identification.

Recent studies (Table 3) have shown that a correct identification by MALDI-TOF is obtained in >80% of cases starting from blood culture bottles. The results varied according to the bacterial pellet preparation protocol and the type of microorganism present in blood cultures. Using ammonium chloride as lysing agent, 89% of Gram-negative bacteria and 73% of Gram-positive bacteria (i.e. 90% for *Staphylococci* and 33% for *Streptococci*) were correctly identified at the species level (Prod'hom*, et al.*, 2010). No identification was observed in 21% of cases. The lower performance of MALDI-TOF for Gram-positive bacteria and particularly *Streptococci* has also been observed in other studies (Table 3). Similar to MALDI-TOF identifications from pure microbial isolates, several hypothesis have been suggested to explain discordant results obtained from blood cultures: i) the close relatedness of the different species especially within *Streptococci*, notably within *Streptococcus mitis* group (i.e., *S. pneumoniae, S. mitis, S. sanguinis, S. oralis*) conferring closely related MALDI-TOF spectrum profiles, ii) the cell wall composition of Gram-positive bacteria conferring resistance to lysis. In addition, the presence of capsules in different species (*S. pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae*) may also explain the lower performance of MALDI-TOF.

The performance of MALDI-TOF for the identification of fungi in blood culture is low. In one study (Ferreira*, et al.*, 2010), no fungi (0/18) were identified at the species level and only one at the genus level. This poor performance is attributed to the relatively low load of fungi observed in positive blood culture and to the presence of residual blood protein which co-migrates during the MALDI-TOF assay, which impairs the performance of the diagnostic algorithm. To circumvent this detrimental effect, a reference database of fungi obtained from blood culture spiked with fungi was established to obtain correct identification at the species level (Marinach-Patrice*, et al.*, 2010).

Limitation of the MALDI-TOF identification was also observed for mixed bloodstream infection, where only one bacterium could be identified (La Scola & Raoult, 2009, Moussaoui*, et al.*, 2010, Szabados*, et al.*, 2011).

The impact of the broth on the spectral quality and thus on the rate of identification has been analysed in several studies. The first published studies have used bottles adapted to the BACTEC system (Becton Dickinson, Franklin Lakes, NJ, USA). More recently, bottles with and without charcoal adapted to Bact/ALERT automated instruments have been tested (bioMérieux, Marcy l'Etoile, France). In this system, charcoal is used to inactivate antimicrobial agents present in patient's blood. In one study, the rate of identification using MALDI-TOF was 30% without charcoal and decrease to only 8% when charcoal was present (Szabados*, et al.*, 2011). Another study compared the performance of identification using MALDI-TOF with positive blood culture obtained from three automated systems: BACTEC, VERSATREK (Trek diagnostic Systems, Cleveland, USA) and BactT/ALERT. The rate of direct identification of bacteria cultured in these 3 automated blood culture devices were 76%, 69% and 62% for the investigated samples, respectively (Romero-Gomez & Mingorance, 2011).

The importance of the protein extraction method was compared with the so-called intact cell method, which consist in the direct deposition on MALDI plate of bacterial pellet obtained from positive blood culture. In one study, the performance of MALDI-TOF identification at the species level was 47% for the intact cell method compared to 76% for the protein extraction method (Ferreira*, et al.*, 2010). The simple extraction method used in this study improves significantly the performance of MALDI-TOF identification rate. In our laboratory, we now use this approach on a routine basis, with a turnaround time estimated to about 1 hour. However, to be efficient and have such a low turnaround time, there is a need to prioritize identification of bacteria isolated from blood cultures over other routine applications of the MALDI-TOF. Thus, this activity somehow delays other microbial identifications, as shown in Figure 4.

In conclusion, the application of MALDI-TOF identification to microorganism pellets obtained from positive blood culture allows a rapid identification of microorganisms growing in blood culture which is important for the management of bloodstream infections.

Urine

Since the bacterial amount in urine taken from patients with urinary tract infection is often $\geq$ 10^5 bacteria/ml, the use of MALDI-TOF directly on urine has been investigated by numerous groups. However, since only about 1 to 2µl of liquid may be deposited on the MALDI-TOF microplate, results were not accurate when untreated urine are directly deposited and thus various pre-treatment steps have been tested with different outcomes. Using two consecutive centrifugation steps (low speed to remove leucocytes and high speed to collect the bacteria), Ferreira *et al.* could accurately identify as much as 94.2% of bacteria (Ferreira*, et al.*, 2010). However, they only included in their study urine with > 10^5 bacteria/ml. When investigating a simplified protocol in our laboratory for the identification of *E. coli* (single centrifugation

step), the results was acceptable when the bacterial concentration was of 10^7 and 10^8 bacteria/ml with 69% and 70% of samples being accurately identified at the species level (score > 2). However, the yield was poor with lower bacterial load (Figure 5). Given the huge amount of urine processed on a daily basis, the low value of early identification and the requirement of bacterial isolation in pure culture for antibiotic susceptibility testing, it appears that the MALDI-TOF on urine is not cost-effective and not efficient enough to be implemented directly on urine samples. Indeed, since most urinary isolates are *E. coli* (> 80% among uncomplicated cystitis occurring in young women and about 50% in complicated urinary tract infections), the use of a chromogenic agar (i.e. Urid, bioMérieux) coupled with simple phenotypic tests such as indole represents a simpler way to identify most strains. MALDI-TOF will then be mainly used to identify the remaining species starting from colonies.

Maintenance and quality controls

The MALDI-TOF MS is increasingly used in clinical diagnostic laboratory for microbial identification with reliable results for bacterial identification at species level.

MALDI-TOF results may however be impaired by problems arising during extraction, for instances (i) when testing encapsulated bacteria (*Streptococcus pneumoniae, Klebsiella pneumoniae*), (ii) when testing bacteria such as *Streptomyces*, that exhibit a particular cell wall that reduces the yield of protein extraction, (iii) when the extraction protocol is not properly conducted, or (iv) when the reagents used for extraction are outdated or impaired by inadequate storage. Problems (i) and (ii), due to intrinsic bacterial properties, may only be circumvented by the development of specific extraction protocols and problems (iii) and (iv) might be prevented by an adequate quality program. The performance of the extraction step and of the MALDI-TOF mass spectrometer may be checked by routinely testing a few

selected bacterial strains, for which spectra are available in the database. This control should ideally be done in parallel with and without a specific extraction step. We thus implemented in our laboratory routine internal quality controls that test the quality of the extraction step on two different bacterial species (*E. coli* ATCC 25922 and *S. aureus* ATCC 25923). To set-up this quality control, we first investigated the reproducibility of the extraction step (see Figure 2 & paragraph on reproducibility). This allowed us to define conserved peaks [peaks present in 10/10 replicates]. Then, we routinely tested once a week both bacterial species. Since score values were always above 2 and to obtain a better expression of the quality of the extraction, we decided to report the proportion of conserved peaks detected, considering a peak as present only when its intensity was above 200. Indeed, the rate of detection of conserved peaks reflects not only the quality of the sample but also the protein yield and the spectral quality (Figure 6).

MALDI-TOF results may also be impaired by inadequate deposit of the sample on the microplate and by poor cleaning of the microplate between runs. Inadequate deposit of samples is relatively rare when starting from bacterial colonies and the learning curve is rapid with most laboratory technicians being already experts in depositing appropriate amount of bacteria after only a few training days. However, erroneous identification may occur due to well inversions, especially when large series are processed and when stress is increased by human resources shortage.

Poor cleaning of the microplate is a problem only encountered by Bruker users since Shimadzu users will use disposable MALDI-TOF plates. Bruker commonly propose to use trichlorofluoroacteate (TFA) or guanidium to clean microplates between usages. Since TFA is associated with significant occupational hazard (eye, skin and respiratory toxicity), we used in our laboratory an alternative protocol in order to clean MALDI-TOF microplate. This protocol, initially proposed by Bruker, which mainly uses ethanol and mechanical cleaning of

target plates, is however insufficient to properly clean MALDI-TOF microplates. Indeed, when investigating cleaned plates by testing them only in presence of matrix, we could obtain some accurate identification with score > 2 and corresponding to the same bacterial species investigated the day before. Such accurate identifications obtained after plate cleaning could rarely correspond to wells where some material was still present (Figure7A, wells C4, E3 and E4), but also occurred in apparently clean wells. Thus, a systematic control of the microplates should be done and the cleaning protocol adapted when necessary. Noteworthy, disposable microplates are now also available for Bruker users.

MALDI-TOF results may also be impaired by technical problems and/or poor maintenance of the MALDI-TOF device. To identify possible technical problems and to recalibrate the mass spectrometry apparatus, we routinely use in Lausanne the calibration control proposed by Bruker. This control (coined BTS) consists of lyophilised *E. coli* extracts and 2 supplementary proteins, RNAse A and myoglobin, which respectively exhibit peaks at 13683 and 16952 m/z (Figure 7B). Finally, in the future, external quality control should be implemented.

Appropriate maintenance (Figure S1) is also essential to warrant accurate bacterial identification. Vacuum failure, and thus MALDI-TOF MS functional disturbance (see figure 1), might be observed due to the presence of dust on plastic joints or to the ageing of these plastic joints (Figure S1 C and D). Dust exposure of the MALDI-TOF apparatus might be reduced by placing the mass spectrometer in a quiet area, without draft. Carbonisation of bacteria embedded in the matrix material following each laser pulse is also a source of concern, since the laser source may be soiled (Figure S1 D). With the Bruker instrument, the rate of dirtiness present on the laser source may be indirectly estimated according to the number of shots needed to obtain a correct identification. Maintenance should ideally be done before the dirtiness rate reach 80%, i.e about 4 times a year if 3 to 5 microplates are tested per

day. Of course, frequency of maintenance of the MALDI-TOF should be increased if the apparatus is heavily used or located in a crowded/dusty area.

Despite adequate maintenance and correct procedures, some microbial groups will repeatedly be misidentified due to poor content of some databases. Thus, it appears critical not only to implement a quality control program targeting routine procedure but also to incrementally improve the quality of the database. In conclusion, quality controls might help to improve the quality of proteins extraction, MALDI-TOF analysis and completeness of databases. This will thus further improve the accuracy and usefulness of MALDI-TOF.

Accuracy, Time and Cost effectiveness

The MALDI-TOF MS approach represents a new tool that has the potential to replace conventional identification techniques for a majority of routine isolates encountered in clinical microbiology laboratories. The performance of the MALDI-TOF MS approach has been compared in several studies (Seng*, et al.*, 2009, Bizzini*, et al.*, 2010, Cherkaoui*, et al.*, 2010, van Veen*, et al.*, 2010) with multiple routine phenotypic identification methods, such as semi-automated Gram staining (Aerospray Wescor® ; Elitech), catalase and oxidase determination tests, automated identification by Vitek ® (bioMérieux), the Api Anaérobie BioMérieux® identification strip for anaerobes (bioMérieux), the Slidex Staph plus system (bioMérieux). These studies showed that the MALDI-TOF technique has a high accuracy for most microbial identifications and performed equally as well as or better than conventional techniques. For instance, van Veen *et al.* showed that significantly more bacterial isolates could be identified to the species level by MALDI-TOF MS, with a special higher performance for staphylococci and bacteria from the HACCEK group (van Veen*, et al.*, 2010). Similarly, Bader *et al.* have shown that identification of yeast species with MALDI-TOF MS systems gave an overall

species identification rate (97.9 % for Bruker and 96.1% for Shimadzu) that was comparable to the one obtained with the biochemical tests (96.9%) (Bader, *et al.*, 2010).

The most striking differences between MALDI-TOF technique and conventional identification methods are observed in the estimated time and costs required for sample identification. The cost of bacterial identification by MALDI-TOF MS was estimated to represent only 17-32% (around €1.43/sample) of the costs of conventional identification methods (around €4.6-8.23/sample) in the study performed by Seng *et al*. (Seng, *et al.*, 2009), which is supported by at least two other prospective studies (Bizzini, *et al.*, 2010, Cherkaoui, *et al.*, 2010). Cherkaoui *et al*. have shown that the reagents required for phenotypic identification using modern automated platforms costs at least around $10 per isolate whereas MS-required reagents do not exceed $0.50 (Cherkaoui, *et al.*, 2010). The expensive prices of MS instruments are comparable to other common bacteriology laboratory equipment such as automated blood culture and 16S sequencing devices but the running costs are significantly cheaper than those of conventional identification methods. Thus, in our laboratory, we estimated that the reagents costs spared during a year are of approximately 40'000 euros (Table S2). Of course, this cost analysis did not include the cost of maintenance, neither for MALDI-TOF nor for automated phenotypic identification systems such as Vitek.

Compared to conventional identification methods, MALDI-TOF has been shown to confer in most cases a significant gain of both technician working time (preanalytical procedure to prepare samples) and turnaround time (automated analytical procedure to obtain results).

The time needed for bacterial identification from intact cells was 6-8.5 min versus 5-48h for conventional identification as estimated by Seng *et al*. (Seng, *et al.*, 2009). When an extraction step is required, Bizzini *et al*. have estimated that the extraction procedure for a single sample takes approximately 6 min, a time per sample that is further reduced during

batch processing (Bizzini, *et al.*, 2010). The time effectiveness gained with MALDI-TOF identification compared to classical identification approaches is even more accentuated when several isolates are analysed in parallel. As reported by Cherkaoui *et al*., the analysis of 10 isolates in parallel can be accomplished in less than 15 min with limiting working time by MS whereas more than 360 min would be required on an automated system with more hands-on time for each isolate (Cherkaoui, *et al.*, 2010) demonstrating that MALDI-TOF MS provide a reduction of both working and turnaround times. Regarding yeast identification by MALDI-TOF MS, Marklein *et al*. showed that this technique required minimal time for technicians to process yeast samples for analysis and to interpret the results (Marklein, *et al.*, 2009). The MALDI-TOF identification procedure from single yeast colonies on the agar plate was generally completed within 10 min per isolate and within 3h for 96 samples. In contrast, the identification of germ tube-negative *Candida* species by phenotypic methods can require incubation periods of up to 72h, a significant longer turnaround time compared to MALDI-TOF. Molecular approaches have been or are currently under development to provide efficient identification of yeasts with a more rapid and reliable efficiency than classical phenotypic methods. However, high-resolution DNA-based molecular techniques such as 26S rRNA or internal transcriber spacer DNA sequencing and real-time PCR assays are expensive and time-consuming and appear in most cases less convenient than MALDI-TOF MS for routine laboratory identifications (Schabereiter-Gurtner, *et al.*, 2007, Montero, *et al.*, 2008, Seyfarth, *et al.*, 2008). Nucleic acid-based identification strategies suffer problematic limitations in terms of (1) technical problems (inhibitory compounds, contamination, separate areas for sample preparation/amplification/analysis), (2) reagent and labour costs, (3) spectrum of species identification in a single assay often limited to a few individual species, and (4) much longer turnaround time than MS. Thus such lengthier, costlier and labour-intensive alternative to MALDI-TOF MS are usually reserved for the identification of the small minority of

isolates not identified by MS alone or by other conventional phenotypic and/or biochemical identification approaches.

Taxonomy and microbial typing

Taxonomic usefulness of MALDI-TOF MS

Taxonomy is the systematic classification of organisms based on their phenotypic, genetic and phylogenetic characteristics. Thus, various phenotypic approaches (morphology, biochemical reactions, and sugar assimilation) have been used by microbiologists to classify microorganisms. However, genome analysis through sequencing of bacterial genes or of the entire genome currently represents the gold-standard of microbial taxonomy, although it should always be confronted to phenotypic traits in a polyphasic approach. MALDI-TOF MS represent an additional approach to classify microorganisms based on phenotypic traits. As previously shown in several studies mentioned in this review, MALDI-TOF MS systems give accurate and reproducible results at the species level that are in most cases concordant with genomic identification methods and consequently, MALDI-TOF has the potential to be used in polyphasic taxonomy. For instance, in contrast to housekeeping genes sequencing such as 16SrRNA that provide taxonomical data on a single gene at a time, MALDI-TOF MS fingerprints provide information about multiple protein components that characterize a microorganism. In addition, MALDI-TOF fingerprints provide data of both the presence (conservation/divergence) and the intensity (expression level) that together compose a two dimensional taxonomical asset which offer a better discriminative resolution for microorganism classification.

Microbial typing and identification at the subspecies level

MALDI-TOF MS allows identification of microbes at the species level and sometimes at the subspecies level but several studies have shown that the requirements for MALDI-TOF MS-dependent microbial typing are different and more complex than those required for routine

microbial identification. This is a challenge for clinical laboratories that want to use MALDI-TOF MS for routine strain typing. Relatively few biomarkers (5 to 10 peaks) are usually required for the identification of microbial isolates at the species level whereas a much larger number of reproducible peaks is needed for subspecies identification (Dieckmann*, et al.*, 2008). Many studies cited in this review using conventional MALDI-TOF procedure could not directly identify taxonomic entities like species or subspecies with an accuracy of 100% without the assistance of DNA-based methods. Microbial typing and thus microbial characterization at the subspecies level required very different sample preparation and analytical procedures (Murray, 2010). As mentioned previously in this review, accurate sample preparation is generally unnecessary for microorganism identification but for strain typing and subspecies identification, a rigorous optimization of testing parameters appears to be crucial. The challenge is to obtain a sufficient number of reproducible markers with specificities below species-level specificity (Rupf*, et al.*, 2005, Vargha*, et al.*, 2006, Dieckmann*, et al.*, 2008). For instance, the sample preparation procedure (whole cell or protein extraction), the protein concentration, the type of matrix, the sample:matrix ratio, the concentration of acid added to the matrix and the growth medium are examples of technical parameters that can have a significant influence on the MALDI-TOF spectral profile of biomarkers (Vargha*, et al.*, 2006, Dieckmann*, et al.*, 2008).

The choice of analysis solutions used to process mass spectra can have a significant impact on the power of discrimination and thus on the ability to distinguish closely related isolates. Maximizing reproducibility is also critical for accurate microbial characterization. One of the major limitations of MALDI-TOF-based microbial typing is primarily due to the algorithmic methods used to analyse the protein profiles. Several similarity coefficients can be used to determinate level of similarities. Some account only for peak presence/absence such as the Dice similarity coefficient, whereas others take also in consideration the peak intensities, such

as cosine and Pearson product-moment correlation coefficients. The chosen similarity coefficient affects the reproducibility and the discriminatory power of the method. Several studies have demonstrated that the Pearson coefficient appears to be more adequate for the correct classification of microbial isolates. A study by Giebel *et al*. showed that the Pearson product-moment correlation coefficient permitted a more accurate classification of *Enterococcus* spp. isolates than the Dice similarity coefficient (Giebel*, et al.*, 2008). Similarly, the use of the Pearson correlation coefficient allowed a 28% increase in the rate of correct classification of *E.coli* isolates (Giebel*, et al.*, 2010). Using optimal sample preparation and MALDI conditions for discrimination at the strain level and by using the Pearson coefficient, Vargha *et al*. have shown that MALDI-TOF MS offered a better discriminatory power than 16S rRNA gene sequencing for the classification at the subspecies level of *Arhtrobacter* isolates (Vargha*, et al.*, 2006). For instance, members of the *A. globiformis* cluster have 99-100% sequence similarity whereas MALDI-TOF MS similarity is 60-95%.

In some cases, the identification of multiple or single unique subspecies biomarkers have been used to discriminate closely related microbial isolates exhibiting highly similar mass signatures. For instance, five unique and conserved biomarkers ions were identified in environmental *E. coli* isolates from avian but not from human sources (Siegrist*, et al.*, 2007). Similarly, several *Listeria monocytogenes* serotypes could be separated using discriminating peaks (Barbuddhe*, et al.*, 2008).

Despite an increased level of complexity required for microbial subspecies classification, several published studies support the observation that MALDI-TOF MS represents a new promising technological approach for the classification of clinical and environmental isolates. Dieckmann *et al*. have successfully classified 126 isolates of *Salmonella* at the species and subspecies levels by optimizing a procedure that allowed them to obtain more than 300

biomarker peaks ranging from 2 to > 35 kDa (Dieckmann*, et al.*, 2008). They found that out of three matrix mixtures, SA produced the most informative spectra by providing a significant increase of high molecular mass peaks with important subspecies specificity. In addition, simple clustering of mass data from bacterial fingerprints did not initially provide a clear discrimination of the strains at the subspecies level and a bioinformatic approach recently published by Teramoto *et al*. had to be used (Teramoto*, et al.*, 2007). The approach is a new phylogenetic classification method based on ribosomal protein profiling by MALDI-TOF MS using the bioinformatics-based method for rapid identification of bacteria published by Demirev and co-workers (Demirev*, et al.*, 1999). Using this approach, the result of the classification of several *Pseudomonas putida* strains including different biovars was in agreement with the *gyrB* gene sequences-based classification.

The determination of serotypes of Shiga toxin-producing *E. coli* isolates has been achieved by constructing prototype spectra representing different serotype groups (Karger*, et al.*, 2011). The prototype spectra were generated by removing masses with low discriminative significance, which is a process comparable to the generation of super-spectra proposed by the SARAMIS software. The generation of prototype spectra allowed a reduction of incorrect assignments down to 0.7% compared to the 31% incorrect assignments observed when unfiltered mass spectra were used. Unlike restriction fragment length polymorphism analysis, this analytical methodology could not achieve a differentiation below the serotype level.

The typing of several microorganisms, such as *Staphylococcus* and *Listeria* species, for epidemiological studies require the use of various conventional techniques such as pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism analysis and multilocus sequence analysis (MLSA). These gold-standard techniques provide accurate classification of microorganism but suffer from important time and cost investments. For instance, only a few hours are required to obtain results by MALDI-TOF MS whereas several

days are necessary to collect PFGE data. In addition, these methods are technically relatively complex and have to be usually performed by experienced technicians.

Several *Staphylococcus* studies have developed standardized methods to achieve reliable and reproducible species level identification and sub-typing from MALDI-TOF fingerprints (Edwards-Jones*, et al.*, 2000, Walker*, et al.*, 2002, Jackson*, et al.*, 2005). When performed under careful experimental conditions, MALDI-TOF MS has been used to discriminate between methicillin-resistant (MRSA) and methicillin-susceptible *S. aureus* strains and to subtype MRSA strains. Thus, compared to conventional antimicrobial susceptibility test methods or gene sequencing techniques, these studies have demonstrated that MALDI-TOF MS represents a fast and cheap approach to accurately differentiate *S. aureus* strains. Unfortunately, no comparison of MALDI-TOF MS with PFGE has been done to demonstrate that these two methods would give similar results. However, Barbuddhe *et al*. have used MALDI-TOF to accurately identify 146 strains of different *Listeria* species and correctly classified all *L. monocytogenes* serotypes in agreement with PFGE, which is one of the most common subtyping technique used to classify *L. monocytogenes* serotypes (Barbuddhe*, et al.*, 2008). Similarly, Fujinami *et al*. have demonstrated that MALDI-TOF MS and PGFE gave similar accurate identification of epidemiologic *Legionella* strains (Fujinami*, et al.*, 2010). Hazen *et al*. have demonstrated that MALDI-TOF MS could be used to discriminate between several *Vibrio parahaemolyticus* strains in replacement of PFGE or multilocus sequence analysis (MLSA) (Hazen*, et al.*, 2009). Thus these studies showed that MALDI-TOF represents a new promising alternative approach to other demanding conventional methods such as PFGE and MLSA for microbial subtyping.

Overall, the ultimate goal would be to use MALDI-TOF for a rapid prospective typing at the time of identification which should significantly benefit to hospital epidemiology and to infection control measures that have to be applied to prevent dissemination of pathogens.

Three recent studies have demonstrated that by applying subtle minor changes in the setup generally used in routine diagnostics, MALDI-TOF MS allowed a reproducible discrimination of major MRSA lineages (Wolters*, et al.*, 2011), an identification of *Salmonella enterica* subsp. *enterica* serovars (Dieckmann & Malorny, 2011) and a differentiation between *cfiA*-negative and *cfiA*-positive *Bacteroidis fragilis* isolates (Wybo*, et al.*, 2011). These studies demonstrate that for several microbial species, minor changes in standardized procedures such as improved algorithm and user-friendly softwares applied in routine diagnostics will allow the use of MALDI-TOF MS for rapid and inexpensive microbial typing. This could significantly improve the approaches currently used to monitor epidemiological outbreaks and pathogens surveillance.

Conclusion

MALDI-TOF represents a very appealing new microbial identification technology that is efficient, rapid, cheap and easy of use. This explains why MALDI-TOF MS can be successfully used in clinical diagnostic laboratory for microbial identification starting from subcultures on agar plates and broth media but also directly from positive blood cultures and to a lesser extent from clinical samples such as urine. The application of MALDI-TOF at the subspecies level in typing is promising but still needs further improvement including instrument sensitivity, database quality and post-run analysis methods. Overall, a MALDI-TOF MS will be soon present in most diagnostic laboratories since despite the significant cost of the instrument and for maintenance, running costs and consumables are much lower than those for other conventional methods rendering this technology a worthy quantum leap tool.

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Tables

Table 1. Performance of routine identification by MALDI-TOF MS.

References	N	Description	Genus level (%)	Species level (%)	Mis-identification (%)	No ID (%)	Comments
(van Veen, *et al.*, 2010)	327	Total	95.1	85.6	3	3.9	• Retrospective intra-laboratory study • Misidentification: associated with absence or mislabeling of reference spectra in the database
	89	*Enterobacteriaceae*	100	96.6	3.4	0	
	55	Non-fermentative GN	81.8	74.5	5.4	14.5	
	87	GP cocci	97.7	80.5	1.1	2.3	
	77	Miscellaneous bacteria (HACCEK)	94.8	84.4	3.9	1.3	
	19	Yeasts	100	94.7	0	0	
(van Veen, *et al.*, 2010)	980	Total	98.8	92	1.7	1.1	• Prospective intra-laboratory study • Misidentification: associated with absence or mislabeling of reference spectra in the database • Viridans streptococci and pneumococci characterized by many misidentifications. • Weakness of the study: lack of sufficient anaerobic bacteria and GP aerobic rods included in the tested samples.
	311	*Enterobacteriaceae*	100	97.7	0.3	0	
	88	Non-fermentative GN	94.3	92	1.1	4.6	
	261	GP cocci in cluster	100	94.3	0.4	0	
	165	GP cocci in chains	98.8	84.8	7.3	1.2	
		Enterococci	100	98.4	0	0	
		Hemolytic streptococci	100	100	0	0	
		Streptococcus milleri group	100	77.8	0	0	
		Pneumococci	100	86.4	0	0	
		Viridans streptococci	90.5	9.5	57.1	9.5	
	94	Miscellaneous bacteria (HACCEK)	96.8	84	0	3.2	
	61	Yeasts	96.7	85.2	3.3	3.2	
(Seng, *et al.*, 2009)	1660	Total	95.4	84.1	1.7	2.8	• Prospective intra-laboratory study • Lack of identification mainly for non-*Clostridium* anaerobes due to an absence of reference spectra in the database
	ND	GP			0.8	2	
	ND	GN			0.9	0.7	
(Cherkaoui, *et al.*, 2010)	720	Total	100	94[a] / 89[b]	0.9[a] / 0.5[b]		• Comparative intra-laboratory study of two commercial MALDI-TOF MS devices (Bruker and Shimatzu) • Bruker system [a] • Shimatzu [b] • Poor yield for streptococcal species and for GN anaerobes (less than 50% correct identification).
	416	*Enterobacteriaceae*		99.8[a] / 95.9[b]	0[a] / 0[b]		
	80	Aerobic GN		97.5[a] / 96.3[b]	1.25[a] / 0[b]		
	111	Staphylococci		98.2[a] / 96.4[b]	0.9[a] / 0.9[b]		
	87	Aerobic GP		73.6[a] / 55.2[b]	3.4[a] / 2.3[b]		
	6	Anaerobic GN		17[a] / 0[b]	0[a] / 0[b]		
	7	Anaerobic GP		57[a] / 43[b]	25[a] / 0[b]		

(Mellmann, *et al.*, 2009)	480 60 60 60 60 60 60 60 60	Total Laboratory A Laboratory B Laboratory C Laboratory D Laboratory E Laboratory F Laboratory G Laboratory H		98.7 100 96.67 100 93.33 100 100 100 100	1.04 0 1.7 0 6.66 0 0 0 0	0.2 0 1.7 0 0 0 0 0 0	• Interlaboratory study • 60 non-fermenting bacteria were shipped to 8 different laboratories with access to Bruker platforms. • No significant difference between direct application and preprocessed samples • Misidentification: sample interchange (4) and skin contamination (1)
(Bizzini, *et al.*, 2010)	1371 1371 525 525 729 729 24 24	Total direct application Total protein extraction GP direct application GP protein extraction GN direct application GN protein extraction Yeasts direct application Yeasts protein extraction	 95.4 99.6 97 100	70.3 92.6 73.7 98.85 71.6 92.2 4.1 100	 4.2 0.95 7 0	 4.6 0.4 3 0	• Intra-laboratory study • Protein extraction increases the total yield of valid results by 25% compared to direct application. • Misidentification: inaccurate taxonomic assignment, change in the taxonomy, limit of resolution of the method.
(Marklein, *et al.*, 2009)	267 267	Total before complementation of database Total after complementation of the database		92.5 100		7.5 0	• Intra-laboratory study of clinical yeast isolates (*Candida* (n=250), *Cryptococcus*, *Saccharomyces*, *Trichosporon*, *Geotrichum*, *Pichia* and *Blastoschizomyces* spp.) • All isolates identified upon complementation of the database with appropriate reference strains. • All samples were preprocessed with a protein extraction step before deposition
(Bader, *et al.*, 2010)	1192 1175[a] / 1152[b]	Total Challenged against respective database		97.6[a] /96.1[b] 99[a] / 99.4[b]	0.7[a]/ 0.2[b] 0.5[a] / 0[b]	1.7[a] / 3.7[b] 0.5[a] / 0.6[b]	• Comparative intra-laboratory study of two commercial MALDI-TOF MS devices (Bruker and Shimatzu) on clinical yeast isolates. • Bruker system [a] • Shimatzu [b] • Better yield observed when the performance is only tested on species present in respective databases • All sample were preprocessed with a protein extraction step before deposition

N, sample number. Genus and species level (%), percent of identification at the genus and species level respectively. Misidentification (%), percent of misidentified samples. No ID (%), percent of samples not identified. GP, Gram-positive. GN, Gram-negative.

Table 2. Problems commonly found in routine identification by MALDI-TOF MS.

Problems	Examples
Limit of resolution of the MALDI-TOF MS method	• *Shigella* spp. identified as *E. coli*
Database discordances • Errors in the reference spectra • Similarities of spectra present in the database[a] • Absence or insufficient reference spectra in the database[a]	• *Propionibacterium acnes* wrongly identified as *Eubacterium brachy* due to incorrect reference spectra in the database • Incomplete reference librairies for viridans streptococci and pneumococci. • No reference of non-*Clostridium* anaerobes in the database. • Insufficient number of reference spectra of *Streptococcus pneumoniae* and *Streptococcus parasanguinis* in the database to differentiate accurately these two closely related species. • Only 1 spectrum of *Propionibacterium acnes* or *Bacillus cereus* present in the database is not enough to be representative of the true diversity of *P. acnes* and *B. cereus* profiles.
Taxonomical discordances	• *Stenotrophomonas maltophila* misidentified as *Pseudomonas hibiscicola,* which is an invalid name for *S. maltophila* • *Agrobacterium tumefaciens* is synonymous of *Rhizobium rhizogenes*
Insufficient protein signal • Difficult to lyse cell wall structures • Small amount of material sample	• Yeasts require a protein extraction procedure to be correctly identified. • Pneumococci as well as most strains of *Haemophilus influenzae* and *Klebsiella pneumoniae* possess a capsule which prevents efficient lysis and results to poor spectral quality. • *Actinomyces*, *Gemella*, *Nocardia* and *Streptomyces* species usually display weak protein signals. • Better signal for *Enterobacteriaceae* grown on blood agar versus Mc Conkey agar

[a] A higher number of reference spectra in the database is usually required to accurately identify closely related microorganisms that display a high degree of spectrum similarities. Thus, these two parameters are interdependent.

Table 3. Performance of MALDI-TOF identification obtained from positive blood culture.

Reference	Sample (n)	Blood culture system	Concordant identification to species level	Concordant identification to genus level	Identification difficulty	Comments
(Prod'hom, *et al.*, 2010)	126	positive blood culture (Bactec)	78%, GN: 89%, GP: 72%	79%, GN: 89%, GP: 73%	*Streptococcus* mitis group, *Staphylococcus* spp.	Use of ammonium chloride to lyse erythrocyte. The presence of a capsule explain partially the low identification rate of *S. pneumoniae*, *H. influenzae*, *K. pneumoniae*
(La Scola & Raoult, 2009)	599	positive blood culture (Bactec)	66% GN : 91% GP : 49%	66%	*Streptococcus* spp., polymicrobial samples	During the study, modification of the extraction protocol to improve *Staphylococci* identification (38% - >75%).
(Stevenson, *et al.*, 2010)	212	positive blood culture (179), spiked bottles (33) (Bactec)	80%	80%	*Streptococcus mitis* group, *Propionobacterium acnes*	Use of a separator device for blood cells removal.
(Ferroni, *et al.*, 2010)	685	positive blood culture (388), spiked bottles (312)	89%	98%	*Streptococcus pneumoniae*, *Streptococcus mitis* group, polymicrobial samples	Use of saponin to lyse erythrocyte.
(Christner, *et al.*, 2010)	277	positive blood culture (Bactec)	94%	95%	Cocci GP	Mismatch mostly resulted from insufficient bacterial quantity and occurred preferentially with GP bacteria

(Ferreira, *et al.*, 2010)	300	positive blood culture (Bactec)	43%, GN: 83%, GP: 32%	72%, GN: 97%, GP: 66%	*Streptococcus mutans*, *Staphylococcus* spp., *Staphylococcus aureus*	No mixed culture
(Ferreira, *et al.*, 2010)	68	positive blood culture (Bactec)	76% ICM 47% PEM 76%	96% ICM 51% PEM 93%	*Staphylococcus* spp.	PEM improve the identification compared to ICM
(Marinach-Patrice, *et al.*, 2010)	48	spiked bottles with *Candida* (Bactec)	100%	100%		Use of SDS as detergent to lyse erythrocytes. Application of a new algorithm's concept since residual blood proteins and *Candida* share many masses in common.
(Szabados, *et al.*, 2011)	268	positive blood culture (BacT/ALERT) (non charcoal containing bottles)	31%	ND	Polymicrobial samples	In a preliminary study, a lower rate of identification with charcoal-containing bottle was observed (8%).
(Romero-Gomez & Mingorance, 2011)	129	positive blood culture Bactec (42) Versatrek (35) BacT/ALERT (52)	68% 76% (Bactec) 69% (Versatrek) 62% (BacT/Alert)	ND	Significant lower performance for GP bacteria	

Adapted from Carbonelle 2010, (Carbonnelle, *et al.*, 2010), GN: Gram-negative bacteria, GP: Gram-positive bacteria, ICM: intact cell method, PEM: protein extraction method, SDS: Sodium Dodecyl Sulfate, ND: not determined.

Figure legends

Figure 1. Technical description of MALDI-TOF MS. The sample is mixed with a matrix on a conductive metal plate. After crystallisation of the matrix and microbial material, the metal plate is introduced in the mass spectrometer and is bombarded with brief laser pulses. The desorbed and ionized molecules are accelerated through an electrostatic field and ejected through a metal flight tube subjected to vacuum until they reach a detector, with smaller ions travelling faster than larger ions. Thus, bioanalytes separated according to their time of flight (TOF) create a mass spectrum that is composed by mass to charge ratio (m/z) peaks with varying intensities. A spectrum is thus a microbial signature that is compared to a database for the identification at the species or genus level.

Figure 2. Intra-laboratory reproducibility tested by measuring the number of conserved peaks in two experimental settings. In the first setting, ethanol/formic acid extraction was applied on *E. coli* ATCC 25922 (A) or *S. aureus* ATCC 25923 (B) and 10 replicates of one extraction were spotted onto the MALDI-TOF microplate (1µl, about 10^6 bacteria/µl). In the second setting, 10 independent extractions were done and one replicate of each extraction was spotted onto the MALDI-TOF microplate. After smoothing and baseline subtraction, the 100 highest peaks from each spot were selected and the number of time a given peak is present out of 10 times was determined for each peak in the two different settings for *E. coli* and *S. aureus*.

Figure 3. The amount of material is a critical factor for accurate microbial identification by MALDI-TOF MS. (A) Spectral fingerprints obtained from various quantities (10^6 to 10^3) of *E. coli* ATCC 25922 grown on blood agar plates. The inoculums were prepared from a sample with a turbidity of 4.0 McFarland that was diluted to obtain the approximate quantity used for direct microplate deposition (10^6 to 10^3 bacteria per spot). Protein extraction was performed by directly mixing the samples with formic acid on microplate. The results show that the quality of the spectrum and thus the performance of identification are largely dependent on

the sample amount spotted on the microplate. (B) Growth of *Staphylococcus epidermidis* on blood agar plates at 35°C in 5% CO_2 atmosphere after an incubation of 2, 4, 6, 8 hours, respectively. Usually, at least 6 hours incubation are required to get sufficient amount of material to obtain an efficient MALDI-TOF MS identification (C) Cumulative percentage of MALDI-TOF identification obtained from Gram-negative (*Escherichia coli* (13), *Pseudomonas putida* (2), *Klebsiella pneumoniae* (2), *Enterobacter cloacae* (1)) and Gram-positive (*Staphylococcus epidermidis* (13), *Staphylococcus aureus* (9), *Streptococcus pyogenes* (4), *Streptococcus pneumoniae* (1), *Staphylococcus hominis* (1)) bacteria after short-time plating on agar during 2, 4, 6, 8 hours, respectively. The number of samples for each bacterial species analysed is indicated in brackets.

Figure 4. Importance of the organization of a working day to optimize time to results. Each line represents a MALDI-TOF run, with the waiting time for equipment availability, the time required to prepare the target layout and the time of the MALDI-TOF running process. In this example of a working day, eight MALDI-TOF target plates have been used to analyse multiple clinical samples (ICU, S: intensive care unit and surgery (1 run); IM, P: internal medicine, pediatry and others (1 target plate); BC: blood cultures (4 runs); U: urine (2 runs)). Urgent samples such as blood cultures are directly processed while colonies identification from agar cultures are processed by batch. The processing of the samples is organized to guarantee the optimal use of the MALDI-TOF device during the working day.

Figure 5. *E. coli* identification yield in urine samples. Five ml of urine samples positive by microscopy were centrifuged to concentrate and collect the bacteria. The pellet was resuspended in 200µl water and subjected to protein extraction with ethanol and formic acid prior deposition on the microplate. The graph shows the identification yield according to the bacterial load per ml.

Figure 6. Importance of the maintenance and quality controls of the MALDI-TOF device demonstrated by a follow-up of the temporal reproducibility. (A) Spectral fingerprint of *S. aureus* quality controls during a 10 weeks control period. (B) Percentage of conserved peaks during a 10 weeks control period. *E. coli* and *S. aureus* quality controls fingerprints were compared to a set of conserved peaks (81 and 80 respectively). The poor performance observed during week 1 to 3 was likely caused by a problem of inadequate sample deposition on the MALDI-TOF MS microplates.

Figure 7. Quality control for MALDI-TOF mass spectrometry. (A) Presence of residual materials on some wells (especially C4, E3 and E4, indicated by white arrows) of a MALDI-TOF micropalte after a routine wash, highlighting the importance of careful wash after each usage; (B) mass spectra obtained with the Bruker BST control, that consists in lyophilized *E. coli* spiked with RNAse A (black arrow at a m/z of 13683) and myoglobin (white arrow at a m/z of 16952); in the lower part of the panel, the list of the eight proteins that should be present to validate the run and their expected and observed m/z values.

Supplementary materials.

Box S1.

Hardwares and Softwares

To date, mainly two MALDI-TOF MS instruments, commercialized by Bruker Daltonics (Bruker Daltonik GmbH, Bremen, Germany) and Shimadzu (Shimadzu Corporation, Kyoto, Japan), are available for routine microbiology. The two companies have different data analysis solutions to process raw MALDI-TOF spectra and to compare this data with spectra of reference libraries. The Bruker instrument provides its own software package, the MALDI Biotyper (Bruker Daltonik GmbH, Bremen, Germany), which includes software and database. The Shimadzu instrument provides the Shimadzu Launchpad software and uses the SARAMIS database (Spectral Archiving and Microbial Identification System, AnagnosTec GmbH, Germany). Both software packages allow processing of raw data (baseline substraction, smoothing, and normalization) and comparison of the processed data to a built-in reference library. The reliability of this technology largely depends on the reference database and the algorithm used for spectral comparison.

The BioTyper reference library currently contains reference spectra for more than 3200 reference strains (Nagy*, et al.*, 2009) and reference spectra for newly investigated bacteria can be added to the reference library (Barbuddhe*, et al.*, 2008, Mellmann*, et al.*, 2008). The library spectra are generated by multiple measurements (average of 20 measured spectra) of known bacterial isolates under slightly different conditions to extract specific peak information. The software automatically generates peak lists from the whole set of spectra and extracts typical peaks which are present in a certain number of spectra from one species.

The SARAMIS database contains over 62,500 single fingerprint spectra of different isolates representing more than 1,160 species, 233 genera and 2,700 super-spectra. Indeed, the SARAMIS database contains two types of spectra: super-spectra and reference spectra. Super-spectra are consensus spectra of multiple mass spectra of reference strains of individual serotypes, species or genera, respectively. A super-spectrum can thus be considered as a reference peak signature of multiple isolates of a species that include a subset of characteristic and reproducible markers (typically 15). The concept of super-spectra has been developed to cope with the natural diversity found in all microbial species which can results in variation of the peaks pattern. The SARAMIS database contains nowadays about 2,700 super-spectra corresponding to 900 different microbial species. Since by definition super-spectra represent the most typical isolates of a species, some less frequent isolates of a given species will not be detected in routine analysis and are then processed using a second-line identification process through a direct comparison to all reference spectra present in the database.

Table S1. Summarized characteristics of several types of mass analysers.

Mass analyser	Advantages	Disadvantages
Time of flight (TOF)	• Unlimited mass range (m/z) • Good mass accuracy and resolution • Simplest mass analyser. Compact and easy to manipulate instrument. • Easily adapted to MALDI. • Low cost.	• Less adapted to ESI than MALDI.
Fourier transform ion cyclotron resonance (FTICR)	• High mass range, up to 10'000 m/z • Excellent mass accuracy and resolution. • Easily adapted to MALDI and ESI • Well suited to analyse complex mixtures	• Expensive, require superconducting magnet.
Quadrupole	• Easily adapted to ESI • Low cost • Small size • Ease to switch between positive and negative ions	• Limited mass range, up to 3000-4000 m/z • Relative low mass accuracy and resolution. Sensitivity and resolution are opposed using quadrupole analysers. • Poor adaptability to MALDI
Ion trap	• Easily adapted to MALDI and ESI • Excellent sensitivity • Ease to switch between positive and negative ions • Low cost • Small size. Simple design.	• Limited mass range, up to 3000-4000 m/z • Relative low mass accuracy and resolution.

Table S2. Costs savings thank to MALDI-TOF MS by reduced need of conventional identification methods. Data derived from a 4 weeks study done in our laboratory, during which a total of 1214 microbial isolates were correctly identified at the species level by MALDI-TOF MS. These costs savings observed on a 4 weeks period were used to estimate the annual costs reduction.

Reagents	Identification	Number	Price/test (€)	Total (4 weeks) (€)	Total (extrapolation 1 year) (€)
Vitek GN ID card	Gram-negative bacteria	270	6.8	1836	23868
Vitek GP ID card	Gram-positive bacteria	146	6.1	890	11570
Rapid ID 32E	*Enterobacteriaceae*	16	5.3	84.8	1102.4
API NH	*Neisseria* and *Haemophilus* spp.	11	7.6	83.6	1086.8
Rapid ID 32 Strep	Streptococci	8	5.3	42.4	551.2
Vitek YST ID card	Yeasts	8	6.1	48.8	634.4
Rapid ID 32A	Anaerobes	1	5.3	5.3	68.9
Api coryne	*Corynebacterium* spp.	1	8.3	8.3	107.9
ID 32C	Yeasts	1	5.3	5.3	68.9
				Total: 3004.5	Total: 39058.5

€, Euro. The prices were converted from Swiss Francs (CHF) to Euros according to the exchange rate of the 19th of April 2011.

Figure S1. Maintenance of MALDI-TOF mass spectrometer: (A) The MALDI-TOF apparatus prior to disassembly. (B) The MALDI-TOF apparatus is completely opened during maintenance to allow inspection and cleaning. (C) Please note the presence of dust particles in the inner part of the MALDI-TOF mass spectrometer (arrow n° 1). (D) Dust may prevent optimal tightness of the plastic joint (arrow n° 2) and this may lead to imperfect vacuum; please note the presence of trace of carbonization at the entry of the acceleration tube (arrow n° 3).

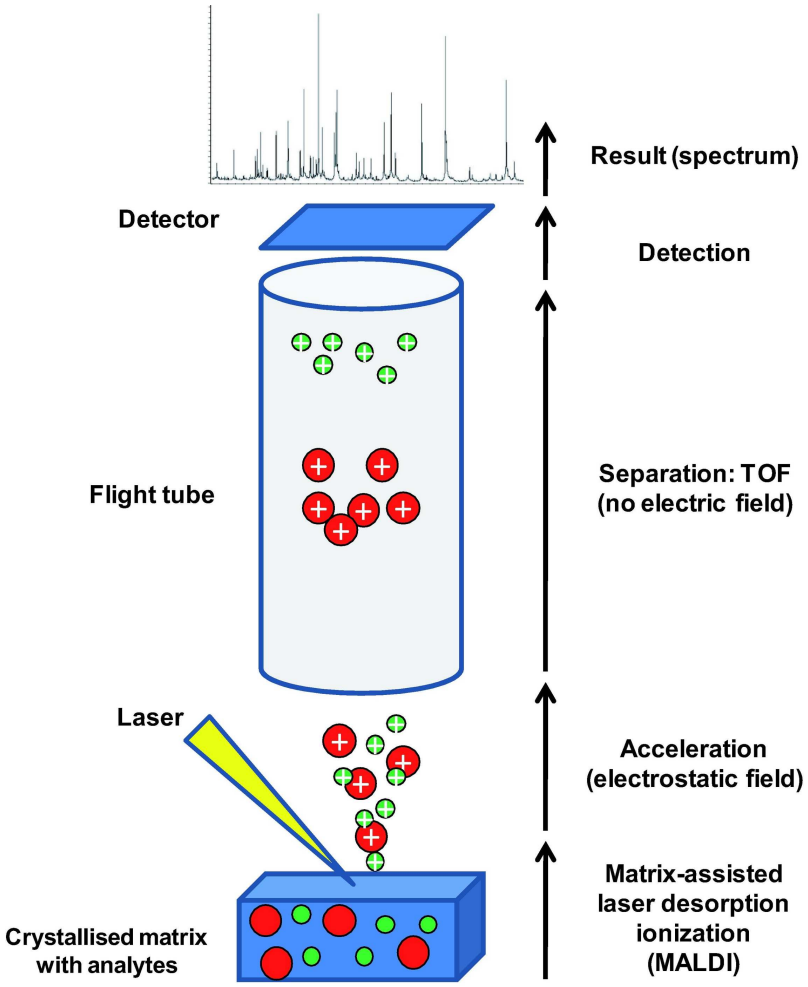

Figure 1

Figure 1. Technical description of MALDI-TOF MS. The sample is mixed with a matrix on a conductive metal plate. After crystallisation of the matrix and microbial material, the metal plate is introduced in the mass spectrometer and is bombarded with brief laser pulses. The desorbed and ionized molecules are accelerated through an electrostatic field and ejected through a metal flight tube subjected to vacuum until they reach a detector, with smaller ions travelling faster than larger ions. Thus, bioanalytes separated according to their time of flight (TOF) create a mass spectrum that is composed by mass to charge ratio (m/z) peaks with varying intensities. A spectrum is thus a microbial signature that is compared to a database for the identification at the species or genus level.
192x289mm (300 x 300 DPI)

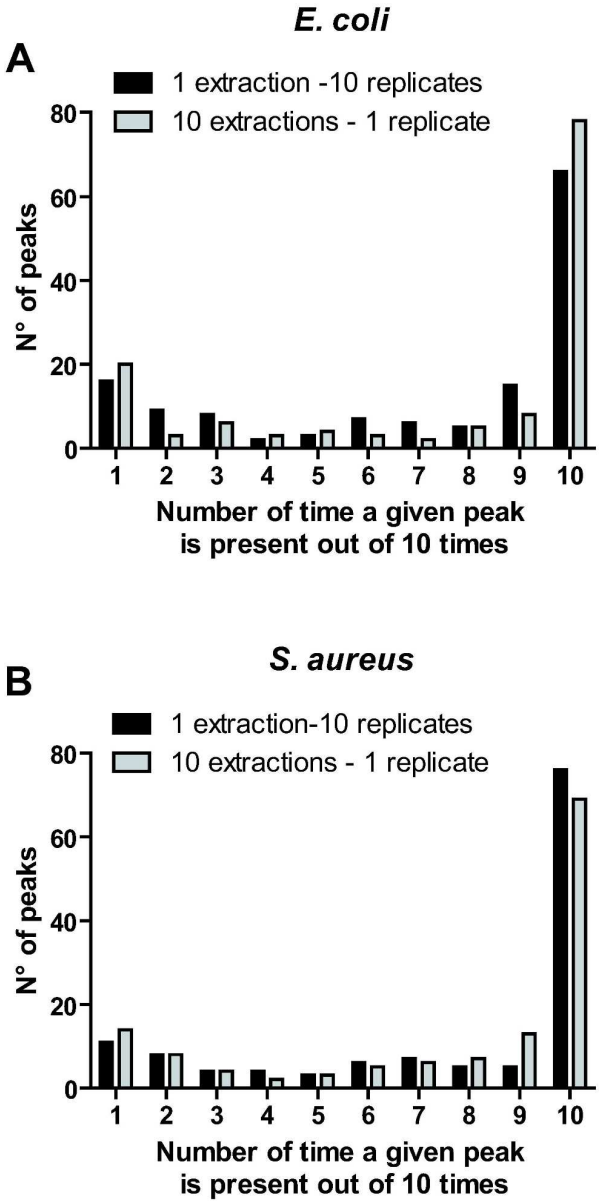

Figure 2. Intra-laboratory reproducibility tested by measuring the number of conserved peaks in two experimental settings. In the first setting, ethanol/formic acid extraction was applied on E. coli ATCC 25922 (A) or S. aureus ATCC 25923 (B) and 10 replicates of one extraction were spotted onto the MALDI-TOF microplate (1 µl about 106 bacteria/µl). In the second setting, 10 independent extractions were done and one replicate of each extraction was spotted onto the MALDI-TOF microplate. After smoothing and baseline subtraction, the 100 highest peaks from each spot were selected and the number of time a given peak is present out of 10 times was determined for each peak in the two different settings for E. coli and S. aureus.
166x335mm (300 x 300 DPI)

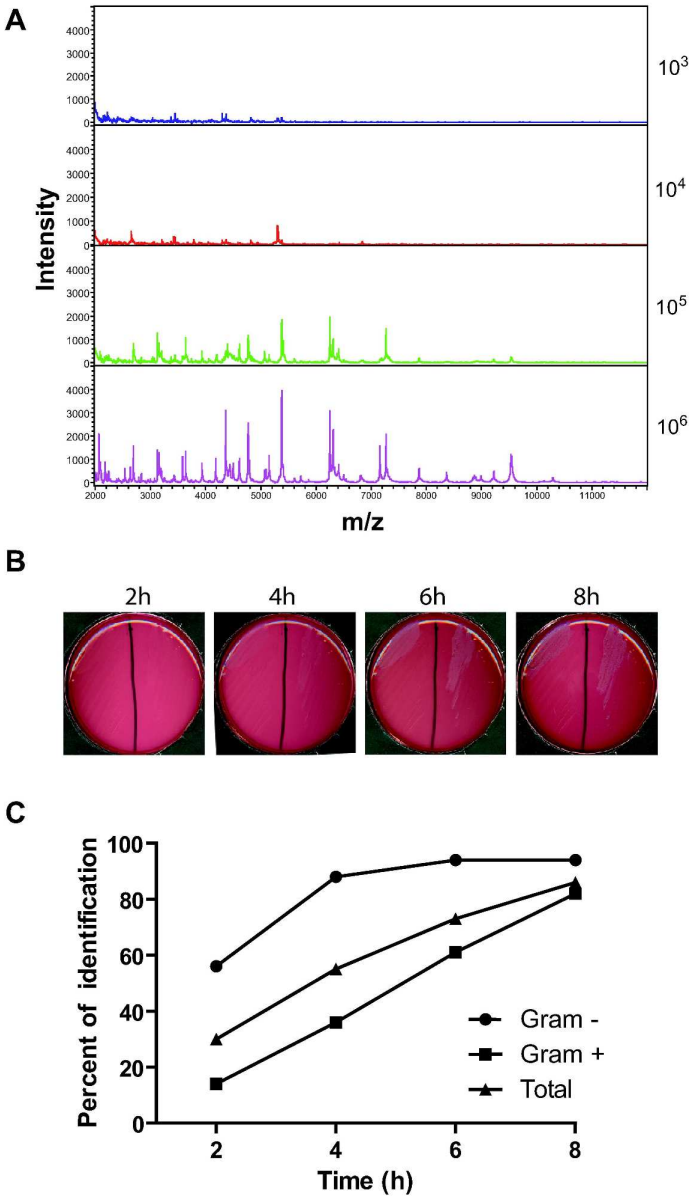

Figure 3. The amount of material is a critical factor for accurate microbial identification by MALDI-TOF MS. (A) Spectral fingerprints obtained from various quantities (106 to 103) of E. coli ATCC 25922 grown on blood agar plates. The inoculums were prepared from a sample with a turbidity of 4.0 McFarland that was diluted to obtain the approximate quantity used for direct microplate deposition (106 to 103 bacteria per spot). Protein extraction was performed by directly mixing the samples with formic acid on microplate. The results show that the quality of the spectrum and thus the performance of identification are largely dependent on the sample amount spotted on the microplate. (B) Growth of Staphylococcus epidermidis on blood agar plates at 35°C in 5% CO2 atmosphere after an incubation of 2, 4, 6, 8 hours, respectively. Usually, at least 6 hours incubation are required to get sufficient amount of material to obtain an efficient MALDI-TOF MS identification (C) Cumulative percentage of MALDI-TOF identification obtained from Gram-negative (Escherichia coli (13), Pseudomonas putida (2), Klebsiella pneumoniae (2), Enterobacter cloacae (1)) and Gram-positive (Staphylococcus epidermidis (13), Staphylococcus aureus (9), Streptococcus pyogenes (4), Streptococcus

pneumoniae (1), Staphylococcus hominis (1)) bacteria after short-time plating on agar during 2, 4, 6, 8 hours, respectively. The number of samples for each bacterial species analysed is indicated in brackets.
200x338mm (300 x 300 DPI)

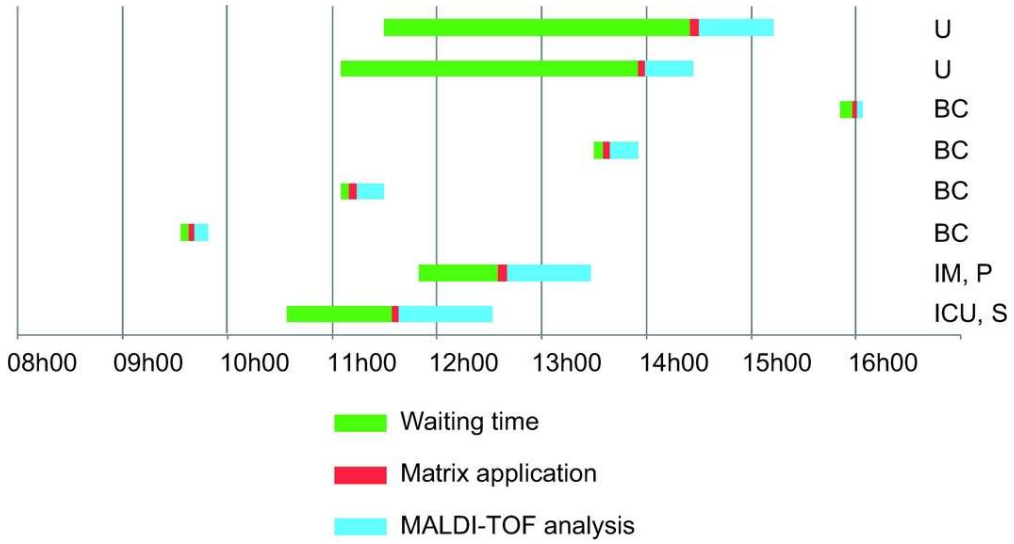

Figure 4. Importance of the organization of a working day to optimize time to results. Each line represents a MALDI-TOF run, with the waiting time for equipment availability, the time required to prepare the target layout and the time of the MALDI-TOF running process. In this example of a working day, eight MALDI-TOF target plates have been used to analyse multiple clinical samples (ICU, S: intensive care unit and surgery (1 run); IM, P: internal medicine, pediatry and others (1 target plate); BC: blood cultures (4 runs); U: urine (2 runs)). Urgent samples such as blood cultures are directly processed while colonies identification from agar cultures are processed by batch. The processing of the samples is organized to guarantee the optimal use of the MALDI-TOF device during the working day.
89x48mm (300 x 300 DPI)

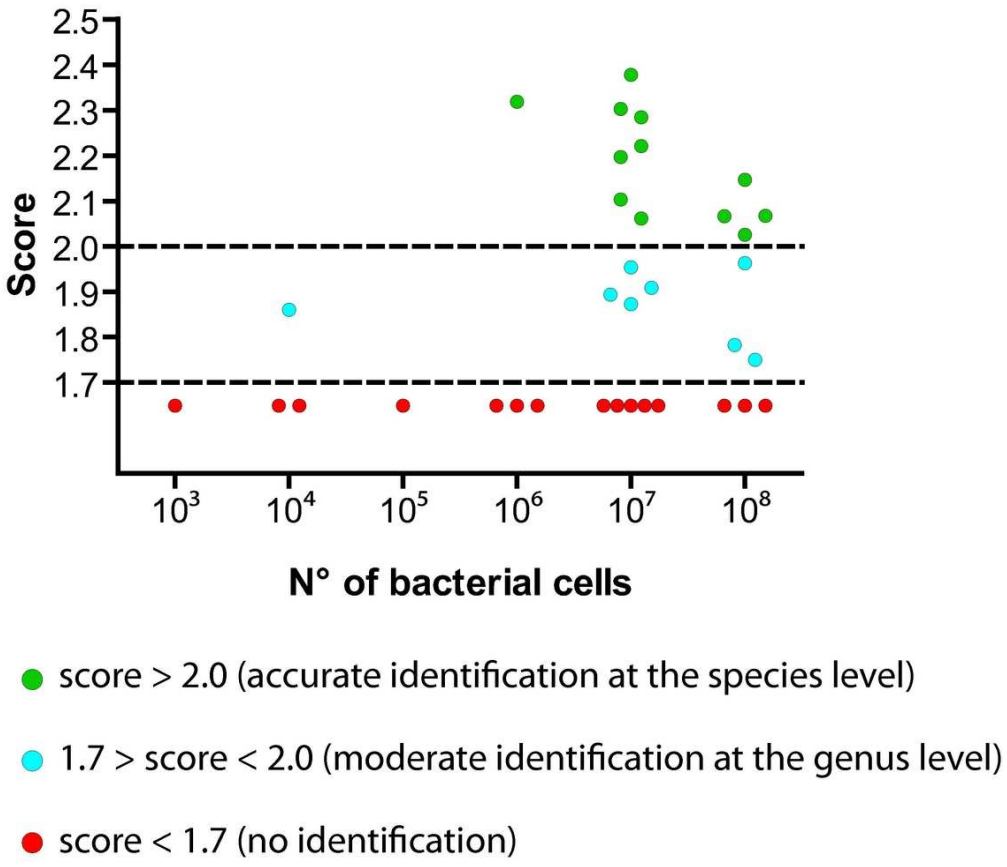

Figure 5. E. coli identification yield in urine samples. Five ml of urine samples positive by microscopy were centrifuged to concentrate and collect the bacteria. The pellet was resuspended in 200µl water and subjected to protein extraction with ethanol and formic acid prior deposition on the microplate. The graph shows the identification yield according to the bacterial load per ml.
95x82mm (300 x 300 DPI)

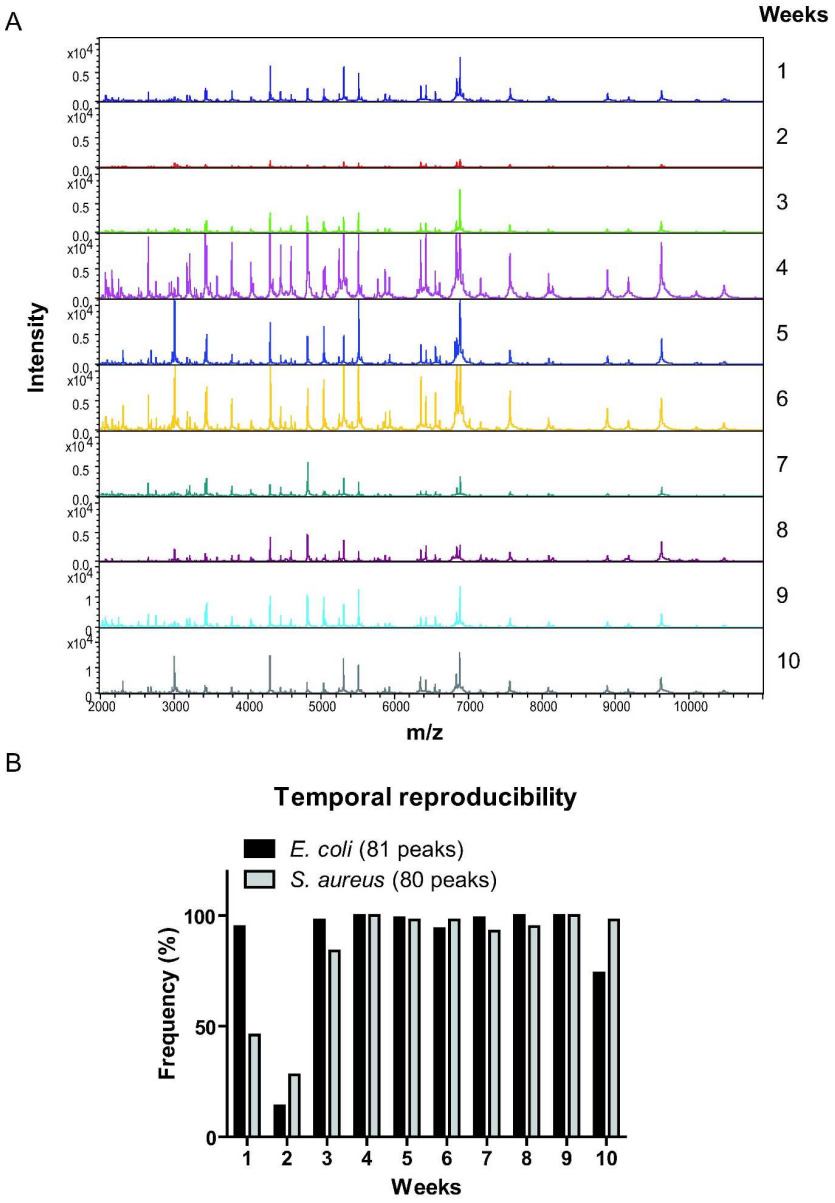

Figure 6. Importance of the maintenance and quality controls of the MALDI-TOF device demonstrated by a follow-up of the temporal reproducibility. (A) Spectral fingerprint of S. aureus quality controls during a 10 weeks control period. (B) Percentage of conserved peaks during a 10 weeks control period. E. coli and S. aureus quality controls fingerprints were compared to a set of conserved peaks (81 and 80 respectively). The poor performance observed during week 1 to 3 was likely caused by a problem of inadequate sample deposition on the MALDI-TOF MS microplates. 229x334mm (300 x 300 DPI)

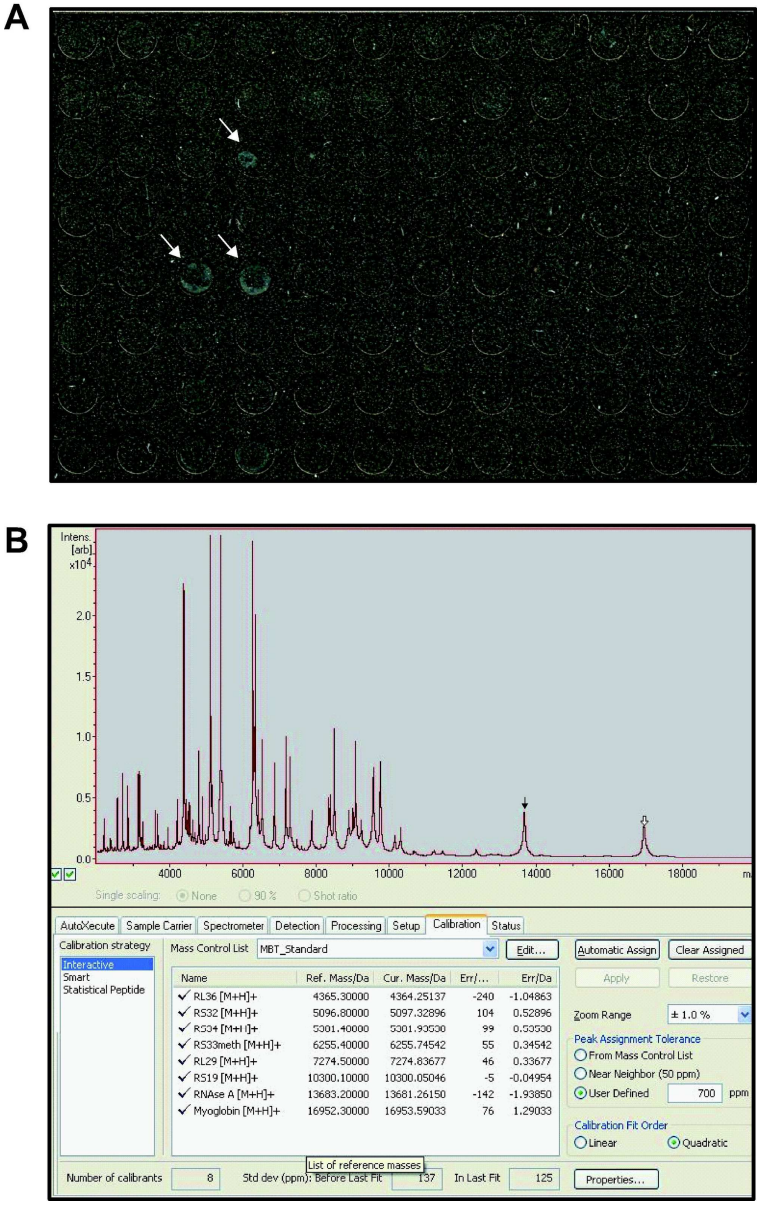

Figure 7. Quality control for MALDI-TOF mass spectrometry. (A) Presence of residual materials on some wells (especially C4, E3 and E4, indicated by white arrows) of a MALDI-TOF micropalte after a routine wash, highlighting the importance of careful wash after each usage; (B) mass spectra obtained with the Bruker BST control, that consists in lyophilized E. coli spiked with RNAse A (black arrow at a m/z of 13683) and myoglobin (white arrow at a m/z of 16952); in the lower part of the panel, the list of the eight proteins that should be present to validate the run and their expected and observed m/z values.
169x269mm (300 x 300 DPI)

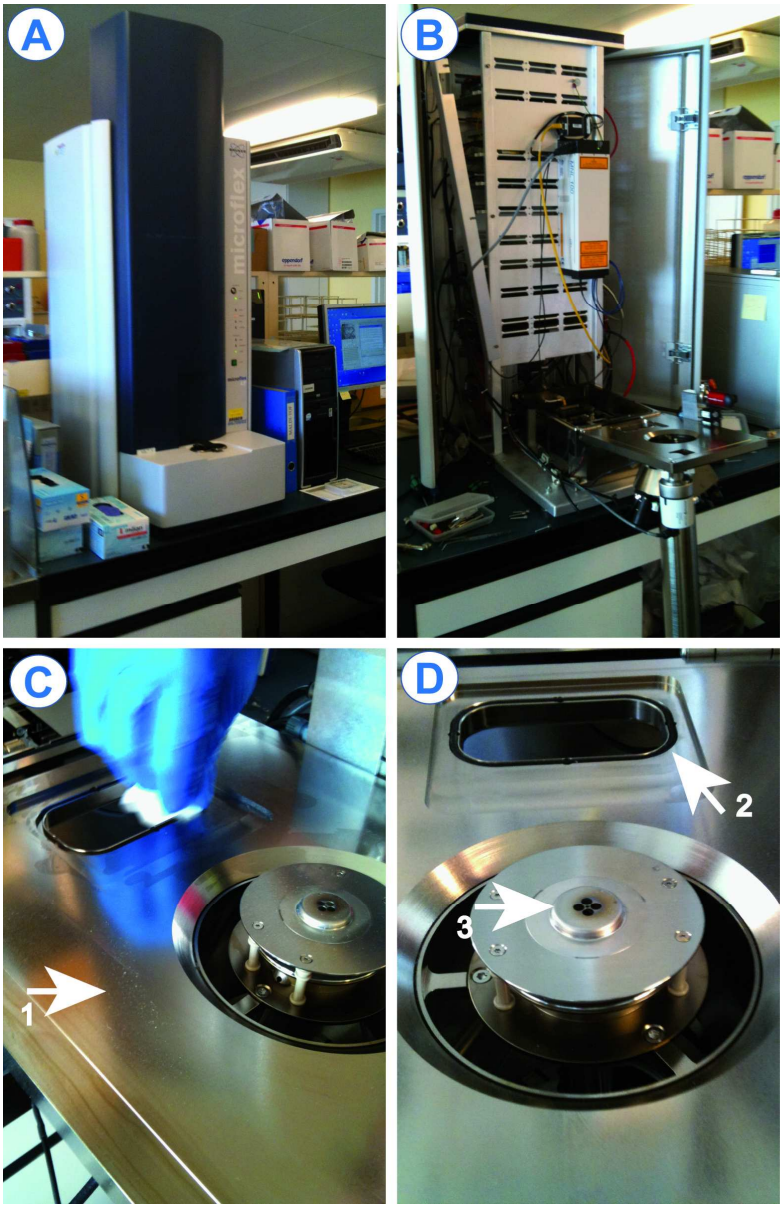

Figure S1.Maintenance of MALDI-TOF mass spectrometer: (A) The MALDI-TOF apparatus prior to disassembly. (B) The MALDI-TOF apparatus is completely opened during maintenance to allow inspection and cleaning. (C) Please note the presence of dust particles in the inner part of the MALDI-TOF mass spectrometer (arrow n° 1). (D) Dust may prevent optimal tightness of the plastic joint (arrow n° 2) and this may lead to imperfect vacuum; please note the presence of trace of carbonization at the entry of the acceleration tube (arrow n° 3).
132x204mm (300 x 300 DPI)