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MALDI-TOF MS: a new laboratory option for the diagnosis of clinical infections

In a recent publication Brundage and Shanks highlighted the importance of secondary infections in viral diseases, using the example of the 1918 influenza pandemic [1]. A large number of deaths, perhaps as many as 30% of the total fatalities, were probably due to bacterial superinfections. This convincingly explains the need for quick and reliable identification of bacterial organisms that are the primary or secondary aetiological agents of infectious episodes.

Standard methods for the identification of microorganisms

Until standardised test systems such as API® and VITEK® 2 (bioMérieux), or PHOENIX® (BD Diagnostics) were developed, identification of clinical microorganisms was based on traditional culture and microscopy methods [2]. With the introduction of these biochemical methods the average time needed to deliver a reliable and validated identification ranged from 6 h to 18 h, depending on the organism isolated. Sequence analysis of small-subunit rRNAs or selected genes by polymerase chain reaction (PCR) methods now complements or in selected cases replaces the biochemical methods [3]. Nucleic acid-based identification methods use stable genotypic characteristics [4]: the bacterial genome contains conserved regions that are particularly suitable for species identification and analysis of phylogenetic relationships [5], and genes encoding virulence factors or toxins provide information on the pathogenicity of a given organism [6–8].

PCR is very useful for detection of viral and bacterial infections. It is a particularly sensitive tool for the detection of noncultivable or slow-growing organisms. Using sequence-specific primers it is possible to detect specifically bacterial DNA without the need for cultivation. DNA sequencing after amplification by PCR is particularly useful for the identification of microorganisms at the specific level, be they bacteria, moulds or viruses. After amplification with nonspecific primers, DNA sequences can be determined and compared with known sequences from databases (e.g., BLAST). DNA sequencing is also necessary for phylogenetic analysis because with it genetic similarities with other microorganisms can be detected and thus robust phylogenetic relationships established [2].

Both biochemical and genetic analyses are reliable, but their use may be time-consuming and their cost comparatively high. There is thus a need for progress towards more rapid and cheap but accurate methods of identifying the aetiological agents of infectious diseases.

MALDI-TOF MS

MALDI-TOF MS (matrix-assisted laser desorption ionisation mass spectrometry – time of flight) is a powerful method for the detection and identification of proteins by molecular weight determination of individual specific fragments. This method is accurate, quick and easy to use, allowing determination of molecular weights with minimal sample requirements [9]. MALDI-TOF MS was developed in the late 1980s by Hillenkamp and Karas, and Tanaka [10, 11]. This made possible the use of mass spectrometry in the analysis of large, nonvolatile molecules such as peptides, proteins and DNA. In MALDI-TOF MS proteins are included in the crystals of UV-absorbing molecules (the so-called matrix). These molecules transfer the acidic protons from UV-absorbing molecules to the analyte molecules by protonisation. In general, the proteins take up one proton and possess a positive charge. In high vacuum, the proteins incorporated in the matrix crystals are UV-irradiated with a pulsing laser and explosively released. The protonated proteins are excreted in the gas phase and fly toward the analyser. Small protein ions reach it faster than the larger ions. This differential time of flight produces a mass-to-charge spectrum.

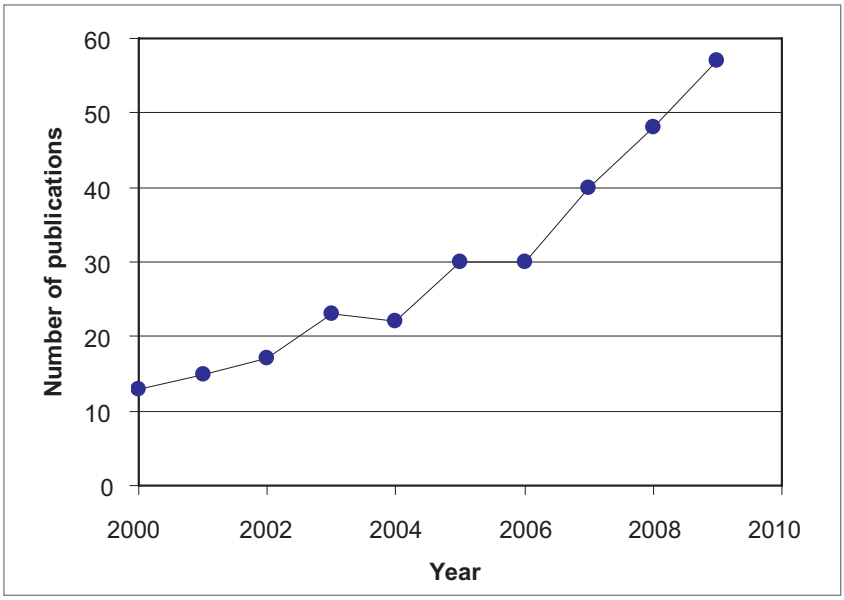

Figure 1
Number of papers referring to the use of MALDI-TOF MS in investigations with bacteria, 2000–2009. Source: PubMed, http://www.ncbi.nlm.nih.gov/pubmed/.

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MALDI-TOF MS is not limited only to the analysis of pure proteins and peptides but can also be applied to intact cells. Generally a fraction of a grown colony or a small quantity of a bacterial suspension is transferred to a stainless steel plate and mixed with the matrix solution. Extracted (chiefly ribosomal) proteins crystallise with the matrix on the target plate, which is then irradiated with a pulsed UV laser. The desorbed molecules are accelerated in an electric field (20 kV) and separated in the TOF device according to their mass-to-charge ratio. Using appropriate tools, their intensity is plotted against their mass-to-charge ratio, thus yielding a species-characteristic fingerprint mass spectrum. This can be compared with existing spectra and the species identified. Handling and sample preparation is straightforward and data analysis quite rapid. In view of the relatively low cost per analysis, the ease of sample preparation and the extremely rapid generation of results, publicly and privately funded microbiology laboratories are increasingly using this technology as the main tool for the identification of bacterial isolates. MALDI-TOF MS has attracted widespread interest in the microbiological community, as shown by the increasing number of publications reporting its use in microbiology. The yearly number of scientific papers dealing with MALDI-TOF MS and bacteria has increased from 3 in 2000 to 350 in 2009 (PubMed citations, search terms MALDI-TOF MS and BACTERIA; fig. 1).

So far MALDI-TOF MS has been developed and validated and is widely used for the identification and characterisation of clinical microorganisms. The currently available databases are targeted at identification of human pathogens [12] and MALDI-TOF MS constitutes a valid alternative to conventional methods of identification and classification of human pathogens in microbiology. Here we present some data generated in our lab and briefly discuss the advantages and disadvantages of MALDI-TOF MS compared to other currently used laboratory methods.

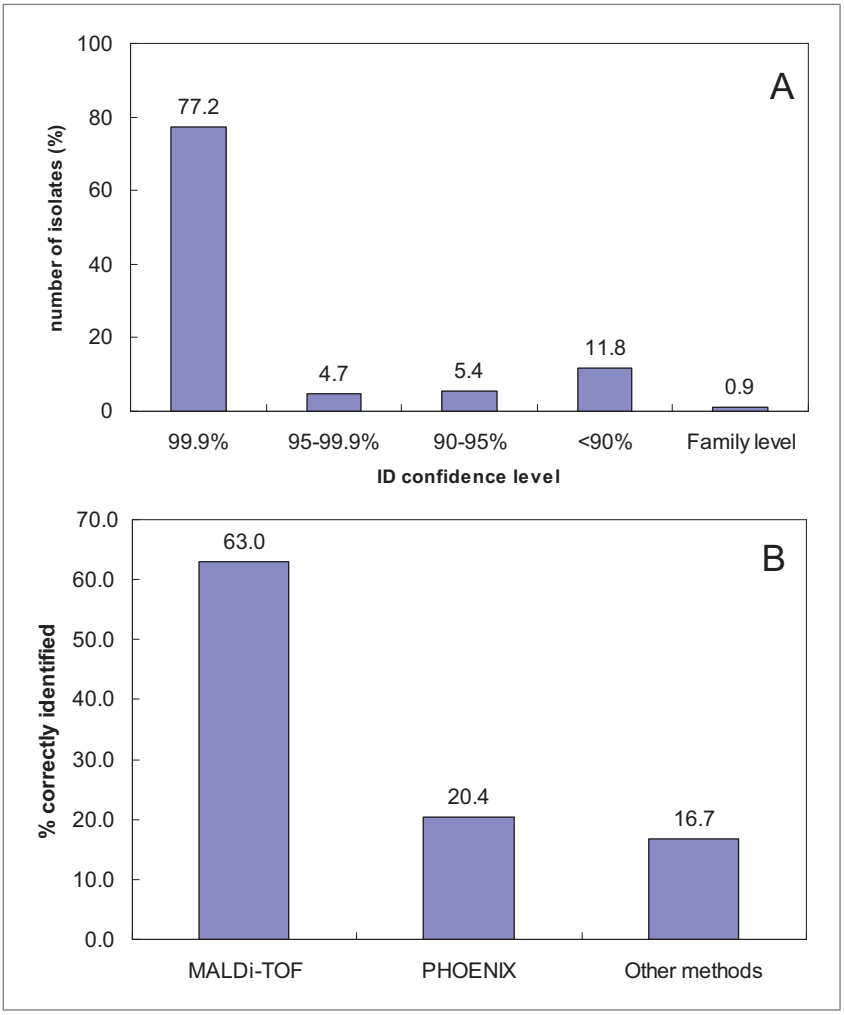

Figure 2
Outcome of the identification of 1021 isolates by MALDI-TOF MS.
2A: Confidence level of identification reported by the SARAMIS database (100%: perfect confidence). Identification confidence levels below 90% indicate the need for confirmation by other methods. 2B. Outcome of identification by 16S sequencing of the isolates for which identification by different methods yielded discordant results. The percentage of isolates with concordance between the molecular method ("gold standard") and any other method is reported.

MALDI-TOF MS in the microbiological laboratory

MALDI-TOF MS in routine clinical diagnostics

A first step in the validation of a new identification system to be introduced in routine diagnostics is to run parallel identifications of a large number of isolates using the new method concomitantly with set standards. In our lab we compared the identification efficiency of MALDI-TOF MS with that of Phoenix®, API® and 16S ribosomal DNA sequence analysis [13].

A small amount of bacterial mass was placed on a steel plate with a plastic loop and overlaid using 0.5 µl 2,5-dihydroxy-benzoic acid (DHB) as a matrix. Plates were analysed in positive linear mode with a MALDI-TOF MS Axima™ Confidence (Shimadzu, Japan). A minimum of 50 laser shots per sample were used to generate each ion spectrum analysed. Spectra were compared with the SARAMIS™ (spectral archive and microbial identification system) database application from Anagnostec GmbH (Potsdam, Germany).

In a first step we analysed 1021 strains obtained from the routine diagnostic lab. For 967 strains (95%) the results of MALDI-TOF MS corresponded to those obtained with the BD PHOENIX system. In approximately 75% of the isolates investigated the ID confidence level was almost 100% (fig. 2A). In 63% of the discordant results the MALDI-TOF MS identification was confirmed

by additional biochemical methods (API®) or PCR/Sequencing. MALDI-TOF was therefore able to identify correctly more than 98% of the isolates tested (fig. 2B).

We then studied 182 clinical isolates including less frequent species belonging to the genera *Staphylococcus*, *Streptococcus*, *Acinetobacter*, *Corynebacterium*, *Pantoea*, and *Gardnerella*. For 138 of them (76%) the identification using MALDI-TOF MS with SARAMIS corresponded to those obtained with PHOENIX, API® or 16S ribosomal DNA analysis. Identification of strains belonging to *Streptococcus* was less reliable, confirming the genetic and phenotypic complexity of this genus.

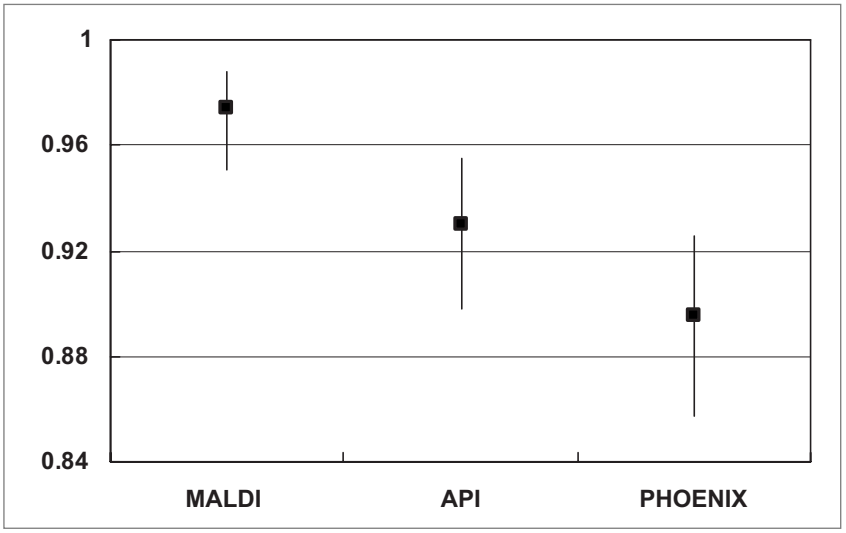

Figure 3
Identification efficiency (computed value and 95% confidence intervals) of MALDI-TOF, API and Phoenix for *S. hominis* as compared to 16S sequencing results (constructed gold standard).

Identification of Staphylococcus species by MALDI-TOF MS

Staphylococci are widespread and feared pathogens in human and animal medicine. We have evaluated the suitability of MALDI-TOF MS in the identification of *Staphylococcus* spp., in particular *S. aureus*, *S. epidermidis*, *S. hominis*, and *S. haemolyticus*, four species commonly isolated from patients in our hospitals.

The study aimed at optimising the sample preparation procedures for these species and testing existing SuperSpectra™ or constructing new ones suitable for the identification of geographical variants. The outcome of identification by MALDI-TOF MS was compared to that obtained by 16S sequencing, which for the purpose of this work was considered the gold standard, and other methods currently used in our laboratory.

In all cases studied, MALDI-TOF MS was at least as good as the other methods in identifying the species studied. The identification agreement between MALDI-TOF MS and the gold standard used, as represented by the Crohn's kappa values, ranged between 0.9 and 0.93, indicating almost perfect agreement. The identification efficiency was also over 90% in all cases (for an example, see fig. 3).

MALDI-TOF MS and fungi

Species of *Candida*, in particular *C. albicans,* are among the most frequent aetiological agents of fungal infections in immunocompromised or debilitated patients. In addition to *C. albicans*, several other species, including *C. parapsilosis, C. dubliniensis, C. lusitaniae, C. krusei, C. glabrata, C. tropicalis* and *C. guilliermondii,* can infect human hosts.

Species identification still relies heavily on conventional morphology, physiology and biochemical characters. More recently molecular methods, including restriction fragment length polymorphism (RFLP), sequencing of internal transcribed spacer (ITS) regions, multilocus sequence typing (MLST) and barcoding have been used to identify clinical and nonclinical isolates [14, 15]. All these methods are relatively costly and time-consuming. We have therefore compared molecular genetic methods with the more rapid and inexpensive MALDI-TOF MS for the identification of *Candida* isolates from human samples [16].

With MALDI-TOF MS all isolates of *C. albicans*, *C. parapsilosis*, *C. magnoliae, C. dubliniensis, C. lusitaniae*, *C. krusei*, *C. glabrata*, *C. tropicalis* and *C. guillier-*

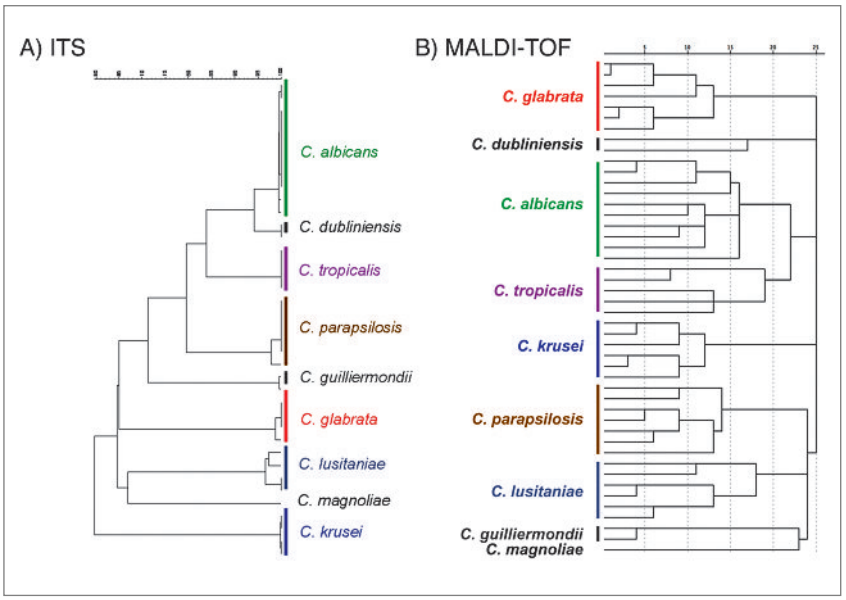

Figure 4
Comparison of the outcome of *Candida* spp. identification by ITS sequencing (A) and MALDI-TOF MS (B).

mondii examined could be reliably identified (fig. 4A). The results were confirmed by genetic analysis (fig. 4B).

Identification of *Candida* spp. by MALDI-TOF MS was identical with that seen with sequence data. Identification by MALDI-TOF MS, however, is more rapid (approx. 5–10 minutes per sample) and cost-effective. For *Candida* spp. DHB was found to be the best matrix. Other matrices tested (sinapinic acid (AS) or α–cyano-4-hydroxycinnamic acid [CHCA]), however, also yielded satisfactory spectra.

Discussion

In our laboratory MALDI-TOF MS has proven to be a fast, accurate and reliable tool for the identification of clinically relevant pathogens, be they bacteria or yeasts. We have observed almost perfect agreement between identifications obtained by MALDI-TOF MS and those provided by conventional, biochemical methods. When discordant results were obtained, sequencing most often confirmed the mass spectrometry identification.

MALDI-TOF MS has also proved very reliable in the identification of filamentous fungi. 129 morphologically and genetically well-characterised strains of *Hypocrea* and *Trichoderma*, belonging to 25 species in 8 phylogenetic clades, were analysed by MALDI-TOF MS. The dendrogram resulting from UPGMA cluster analysis of MALDI-TOF MS data and phylogenetic trees produced with ITS and *tef1* sequences were roughly identical and confirmed previously defined clades and sections in the genus [17]. Work is currently ongoing in our lab to validate MALDI-TOF MS analysis for the identification of dermatophytes and other filamentous human pathogens.

MALDI-TOF MS, however, is not the magic bullet. For instance, reliable identification of organisms by this technique is only possible if the strains used to construct the spectral database have been carefully selected and identified by reliable methods, i.e., ideally, sequencing of characteristic gene regions (e.g. 16S RNA *gyrB*, *rpoB*, or *hsp60* genes for bacteria and ITS regions for fungi). Careful calibration of the machine and selection of adequate internal standards is also needed, in particular when intraspecific characterisation is needed. Geographic variation in the genotypic and phenotypic expression of some bacteria (e.g., *Streptococcus* or *Staphylococcus*) may require region-specific calibration and preparation of locally adapted databases. Finally, care should be taken always to use the culture medium that has been used to construct the reference spectra.

Table 1 summarises strengths and weaknesses of MALDI-TOF MS compared to other systems in use. Overall, MALDI-TOF has found a well-deserved place in the armamentarium of bacterial and fungal taxonomists. Problems with the analysis of mixed cultures, currently not yet completely solved, are likely to be overcome in the near future, and attempts to identify

Table 1 MALDI-TOF MS compared to other identification methods

	Strength	Weakness
IC MALDI-TOF MS based identification	**Quick and simple** – Starting from bacterial colony the result can be obtained within a few minutes – Easy sample preparation – High throughput **Cost-effective** – Cost of MALDI-TOF apparatus comparable to cost of medium size sequencer, but running costs (consumable) are only a fraction thereof **Reliable identification** – Down to species or in individual cases even to subspecies and strain level – Database-related problems can be overcome by including an increasing number of species or by providing, within a particular study, reference samples for comparison	**Cultivation-dependent** – Colonies/growth must be obtained in order to perform analysis **Fingerprinting-based method** – Works only in comparison with a reference **Identification limited by database** – Most of the bacteria currently included in the database are clinically relevant species – Environmental species are underrepresented
Biochemical profining	– Numerical taxonomy – Automation possible – Several commercial systems available – Easy standardised operations	**Cultivation-dependent** **Identification limited by database** – Database well-developed for bacteria, but not for other kingdoms – Most of the bacteria currently included in the database are clinically relevant species – Environmental species are underrepresented
Sequencing of genetic markers	– Genetically-based identification method – Large public databases of reference sequences – Cultivation-independent	– High cost – Time-consuming – Different genetic markers

pathogens directly in the clinical samples have already yielded satisfactory results [18]. There are already some hints that MALDI-TOF MS may be used in susceptibility testing of fungi and bacteria [19]. Economically, the high acquisition costs of the machine are compensated by the savings made with consumables and maintenance. From the technique's ease of use, coupled with the rapid and reliable identification it provides, MALDI-TOF MS can be expected to become the method of choice for identification of clinically and environmentally relevant organisms.

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