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# Species Identification of Bacteria and Fungi from Solid and Liquid Culture Media by MALDI-TOF Mass Spectrometry

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## Abstract

Within the past years MALDI-TOF mass spectrometry has become a powerful tool for the species identification of cultured bacteria and fungi. In the present study, the implementation of MALDI-TOF mass spectrometry in a commercial high throughput laboratory is described. The impact of different growth conditions on the identification results was evaluated. Although slight differences in MALDI-TOF spectra of *E. coli* and *S. aureus* strains cultured on blood agar for various periods (5, 18, 24 and 48 hours) were noticed, reliable species identification was obtained for all periods. The same was true when *E. coli* and *S. aureus* strains were cultured for 18 hours on various solid media. Reliable identification was also achieved when fungi were cultured on solid and in liquid media (Sabouraud bouillon). Moreover, growth of fungi in bouillon resulted in accelerated identification. MALDI-TOF mass spectrometry also allowed reliable identification of microorganisms from positive blood culture samples. In total, 2,900 specimens (234 different species) predominantly derived from clinical samples were examined. Microorganisms were cultured on solid media, in blood culture bottles and in liquid Sabouraud bouillon. 98.6% (n=2,860) of the MALDI-TOF identification results matched those of conventional methods (e.g. Gram staining, carbohydrate degradation ability, Phoenix system) and 16S rDNA PCR product sequencing. Mismatches were mainly based on missing reference spectra in the database of the analysis system. The study was performed in 2009. Due to continuous improvement of the database, even higher accuracy would be achieved when performing the study nowadays. In summary, the use of MALDI-TOF mass spectrometry in a clinical high throughput environment leads to reliable results, even when various culture conditions are used, for instance bouillons for culture of fungi. MALDI-TOF mass spectrometry is a fast and robust identification system, which is on its way to become a new standard for the identification of microorganisms in high throughput laboratories.

**Abbreviations**: CHCA: Alpha-Cyano-4-Hydroxycinnamic Acid; CNA: Colistin / Nalidixic Acid; ESI: Electrospray Ionization; MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; m/z: Mass to charge ratio; TFA: Trifluoroacetic Acid.

## Introduction

For more than one hundred years after the death of Robert Koch (May 27th, 1910) microbiological diagnostic remained similar. As it used to be, species identification is predominantly based on morphological aspects (e.g. Gram positive vs. Gram negative) and the ability of microorganisms to metabolize certain energy sources (e.g. carbohydrates and lipids). Consequently, identification of bacterial and fungal species has only been slightly accelerated within the past hundred years and usually lasts several days.

The first attempts to analyze large biomolecules using mass spectrometry (MS) were already performed in the 1970's [1]. Almost at the same time, first experiments were carried out to identify microorganisms by mass spectrometric methods [2]. However, due to hard ionization techniques, extended fragmentation of the analytes was received, and therefore it was not possible to generate reliable spectra. At the end of the 1980s, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) were developed allowing gentle ionization of large biomolecules (>250,000 Da) and in 2002 the Nobel Prize in chemistry was awarded to John B. Fenn (ESI) and Koichi

Tanaka (MALDI). With the discovery of MALDI, reproducible analysis of large biomolecules (e.g. ribosomal proteins) and consequently the differentiation between bacterial species was enabled [3–7]. MS based species identification can be performed within a timeframe of a few minutes while conventional analysis takes several hours and is usually performed on overnight cultures.

### Physical background

There are several excellent sources which describe the technical basis of MALDI-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) in detail [8,9]. Since this technique is still a rather new method for the identification of microorganisms in clinical laboratories, a brief overview is given here.

Before analysis, sample material is transferred to a small metallic

plate (MALDI target). This plate comprises up to 384 sample places. Microbiological samples commonly consist of cultured bacteria, but fungi may also be examined. Furthermore, microorganisms from liquid materials (urine, blood cultures, etc.) can be analyzed by MALDI-TOF MS. After transfer to the MALDI target plate, samples are overlaid with a drop of an organic acid, which then is air-dried. The crystallized acid forms the "matrix". The use of such a matrix in the form of small, laser-absorbing organic molecules in large excess over the analyte is the core of the MALDI principle. Various organic acids are suitable for the MALDI-TOF procedure. While gentisic acid is adapted for analysis of small molecular weight components, sinapinic and cinnamic acid (usually alpha-cyano-4-hydroxycinnamic acid, CHCA) are adequate for examining proteins [10]. The matrix solution also contains a solvent, for instance acetonitrile, which leads to co-crystallization of matrix and analyte molecules when evaporating.

After the transfer of the sample plate into the high vacuum system of the spectrometer, a laser pulse of a few nanoseconds is applied to the sample, hence the plate is commonly called a target. The laser pulse leads to ionization and desorption of bacterial proteins. In comparison to sample material, the amount of matrix is much higher (1,000-10,000 fold excess), causing laser energy to be absorbed almost entirely by the matrix. Uptake of laser energy causes excitation of matrix molecules (pressure pulse) leading to micro-explosions and subsequently to a release of matrix and bacterial molecules from the target (desorption), followed by the ionization of the molecules. However, more than 20 years after the invention, the overall process of desorption and ionization has not yet been fully described. One theory implies that ionization takes place during the release of sample molecules. Alternatively, ionization may occur during co-crystallization of matrix and analyte molecules. However, in the course of desorption, the majority of ionized proteins are deionized and only a few ionic sample proteins, the so-called "lucky survivors" remain [11,12].

An electric potential in the range of 20 kV is applied to an electrode within the spectrometer leading to an acceleration of ionized proteins towards the detector. After passing the electrode, ionic speed remains constant until the ions are recorded by the detector. The vacuum within the spectrometer prevents collisions of the ions with air molecules. Therefore, velocity of the ions depends only on mass and charge, represented by the term mass to charge ratio (m/z). Fortunately, the MALDI process almost entirely results in the formation of single charged ions. Consequently, the time-of-flight basically depends on the mass of the ions. To increase discriminatory power, the induction of laser pulses and application of the acceleration voltage are synchronized allowing the determination of the time-of-flight, i.e., the interval between ions passing the electrode and reaching the detector (Figure 1).

## Management of recorded spectra

The x-axis of a MALDI-TOF spectrum shows the m/z ratios of detected proteins, while the intensity representing the amount of ions is demonstrated on the y-axis (Figure 2).

The two largest companies providing MALDI-TOF MS systems in the field of microbiology (Bruker, bioMérieux) use different protocols for the interpretation of a recorded spectrum. The Bruker Main Spectrum analysis compares a newly recorded spectrum to those of deposited reference spectra. The bioMérieux system compares newly recorded spectra to so-called "Super Spectra" created from deposited reference strains and also from spectra of clinical isolates [13]. The use of superspectra is based on the idea that not each peak of a spectrum is relevant for identification of the respective species. Therefore, a
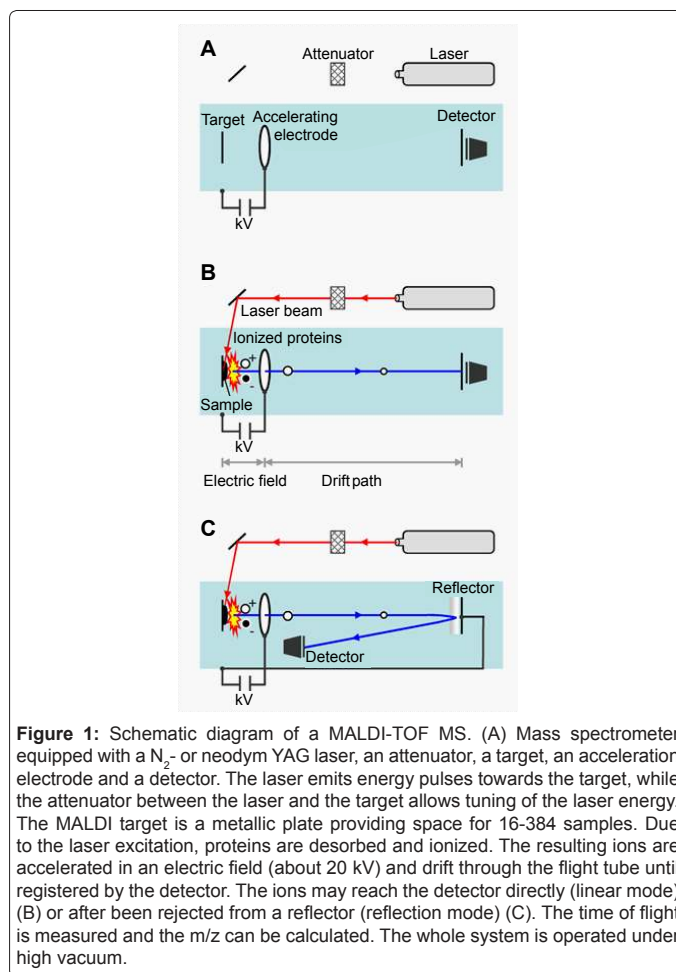


**Figure 1:** Schematic diagram of a MALDI-TOF MS. (A) Mass spectrometer equipped with a N$_2$- or neodym YAG laser, an attenuator, a target, an acceleration electrode and a detector. The laser emits energy pulses towards the target, while the attenuator between the laser and the target allows tuning of the laser energy. The MALDI target is a metallic plate providing space for 16-384 samples. Due to the laser excitation, proteins are desorbed and ionized. The resulting ions are accelerated in an electric field (about 20 kV) and drift through the flight tube until registered by the detector. The ions may reach the detector directly (linear mode) (B) or after been rejected from a reflector (reflection mode) (C). The time of flight is measured and the m/z can be calculated. The whole system is operated under high vacuum.
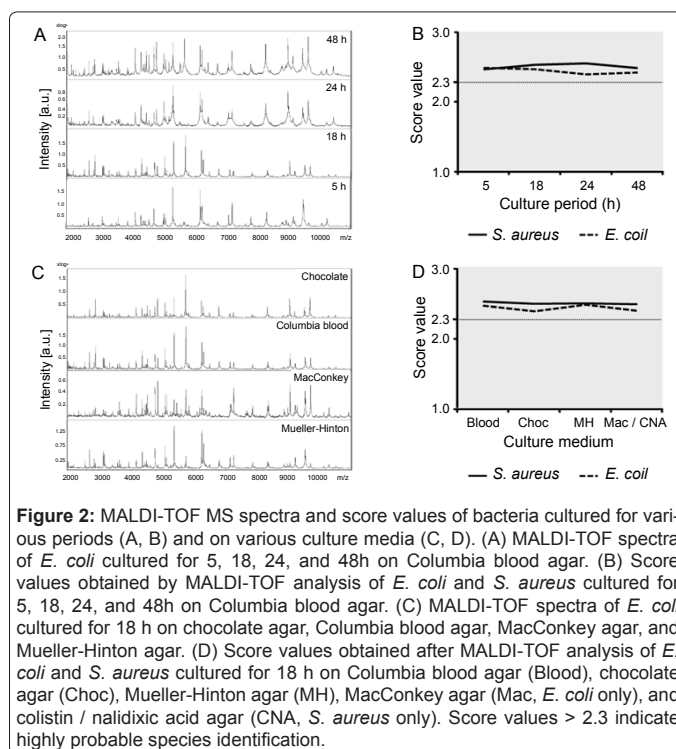


**Figure 2:** MALDI-TOF MS spectra and score values of bacteria cultured for various periods (A, B) and on various culture media (C, D). (A) MALDI-TOF spectra of *E. coli* cultured for 5, 18, 24, and 48h on Columbia blood agar. (B) Score values obtained by MALDI-TOF analysis of *E. coli* and *S. aureus* cultured for 5, 18, 24, and 48h on Columbia blood agar. (C) MALDI-TOF spectra of *E. coli* cultured for 18 h on chocolate agar, Columbia blood agar, MacConkey agar, and Mueller-Hinton agar. (D) Score values obtained after MALDI-TOF analysis of *E. coli* and *S. aureus* cultured for 18 h on Columbia blood agar (Blood), chocolate agar (Choc), Mueller-Hinton agar (MH), MacConkey agar (Mac, *E. coli* only), and colistin / nalidixic acid agar (CNA, *S. aureus* only). Score values > 2.3 indicate highly probable species identification.

superspectrum only contains a set of relevant species-specific peaks. If a recorded spectrum does not match a superspectrum, it is compared to peaks of full spectra from the database. Both systems inform the user in the diagnostic laboratory which species matches the recorded spectrum best. In the Bruker system the reliability of this assignment is expressed by so called score values while the bioMérieux system gives percent values.

Beside proteins, various other substrates (e.g. carbohydrates, nucleic acids) might theoretically be examined by MALDI-TOF MS. However, the concentration of these substances is frequently low, e.g. that of DNA. Therefore, examination of proteins is more promising than examinations of other cellular components. Especially ribosomal proteins are suitable biomarkers for this analysis because cellular concentration is high and specific posttranslational modifications result in varying masses making ribosomal proteins distinguishable by MS [14]. However, ribosomal modifications due to altered growth conditions might hamper the species identification. Therefore, the impact of growth conditions on the reliability of spectra composition must be examined before switching species identification from conventional methods to MALDI-TOF MS.

## Aim of the Study

In the present study we show the implementation of MALDI-TOF MS for routine diagnostics in a large microbiological laboratory in southwestern Germany. We demonstrate the suitability of a MALDI-TOF MS system for the identification of species from pure bacterial and fungal colonies, as well as for fungal species from pure liquid cultures and bacteria from positive blood cultures.

## Material and Methods

### Cultures media

Agar plates for bacterial growth were obtained from Becton Dickinson, Heidelberg, Germany (Mueller Hinton agar, blood and chocolate agar, blood agar supplemented with Colistin-Nalidixic Acid (CNA), MacConkey agar) and from BioMerieux, Nürtingen, Germany (MRSA chromogenic agar - chromID™).

For fungal growth Sabouraud agar (Becton Dickinson) and BBL™ CHROMagar™ Candida (Becton Dickinson) was used. Dermatophytes were cultured on Mycosel™ agar (Becton Dickinson) and differentiated on potato dextrose agar (Oxoid, Wesel, Germany). For production of Mycosel™ and potato dextrose agar powder had been obtained from the companies and prepared according to the manufacturer instructions.

### Bacteria

Initially, *Escherichia coli* and *Staphylococcus aureus* strains isolated from patients were cultured for varying durations (5 h, 18 h, 24 h and 48 h) to examine if different bacterial growth time alters composition of MALDI-TOF MS spectra. *E. coli* and *S. aureus* were also cultured for 18 h on various solid media (Mueller Hinton agar, blood and chocolate agar, CNA, MacConkey agar) to examine the impact of culture medium on the generated spectra. The recording of MALDI-TOF MS spectra and particular score values depend only subordinately on culture conditions [15,16]. This allows the use of appropriate media (selective and non selective) for the cultivation of microorganisms in this study. In total, 2,653 clinical isolates were collected in the Medical Care Centre Leinfelden within a six month period. These isolates originated from the whole spectrum of microbiological sampling available for human health care. Bacterial colonies were recovered after

aerobic, microaerophilic and anaerobic incubation overnight or, in case of slowly growing species, for several days. Isolates were identified through standardized cultural, morphological and biochemical (API system, Biomérieux, Nürtingen, Germany) criteria.

### Fungi

Exclusively fungal isolates allowing valid species determination participated in the study. In total, 247 isolates (184 yeasts, 39 dermatophytes, 24 molds) were collected and analyzed during the six month period. Yeast isolates were cultured on chromagar (BD, Heidelberg, Germany) and characterized by the API ID 32C (Biomérieux, Nürtingen, Germany) after the manufacturer's recommendation.

Dermatophytes and molds originated from clinical specimens collected in the Synlab laboratory Leinfelden (n=16), the University Hospital of Zürich (CH), Department of Dermatology (n=30) and German INSTAND interlaboratory control panels (n=17). Among those were *Aspergillus candidus* (n=1), *A. flavus* (n=1), *A. fumigatus* (n=2), *A. glaucus* (n=1), *A. niger* (n=1), *A. terreus* (n=4), *A. versicolor* (n=3), *Aureobasidium pullulans* (n=1), *Chaetomium globosum* (n=2), *Epidermophyton floccosum* (n=2), *Exophiala dermatitidis* (n=1), *Geotrichum candidum* (n=1), *Microsporum audouinii* (n=5), *M. canis* (n=3), *Paecilomyces lilacinus* (n=1), *P. variotii* (n=2), *Prototheca wickerhamii* (n=1), *Rhizopus microsporus* (n=1), *Scopulariopsis brevicaulis* (n=3), *Sporothrix schenckii* (n=1), *Trichophyton ajelloi* (n=2), *T. rubrum* (n=16), *T. schoenleinii* (n=1), *T. tonsurans* (n=1), *T. violaceum* (n=1) and *Trichosporon mucoides* (n=1). Fungi growing deeply into the agar medium were cultured in parallel on solid agar and in liquid media (Sabouraud bouillon; BD, Heidelberg, Germany). All dermatophyte and mold samples were tested in duplicates with MALDI-TOF MS. Dermatophytes and molds were evaluated macroscopically and microscopically according to standardized criteria as previously described [17,18].

### Blood cultures

23 blood culture samples (21 aerobe, 2 anaerobe) (BACTEC™ blood culture media, BD, Heidelberg, Germany) collected from patients with suspected sepsis identified as positive by the BACTEC™ blood culture system were collected and analyzed by MALDI-TOF MS (Table 1). All positive blood culture bottles per patient were tested and samples were analyzed in duplicates.

### Sample Preparation

**Protocol 1:** For routine use, all bacterial and yeast samples were smeared on the MALDI target as a thin film and overlaid with 1.5 µl of formic-acid (70%), air-dried and overlaid with 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 2.5% TFA).

**Protocol 2:** An ethanol/formic acid-extraction procedure [16] was used when identification results received with protocol 1 were (i) not reliable (score value < 1.7) or (ii) resulted in ambiguous identification compared to conventional methods. Furthermore, the protocol was used for the preparation of all dermatophyte and mold specimens.

Single colonies were suspended in 70% ethanol, spun down, resuspended in 30 µl of 70% formic acid followed by addition of 20 µl of 100% acetonitrile. After centrifugation, 1 µl of the supernatant was transferred on the MALDI target, air-dried and overlaid with matrix solution.

**Protocol 3:** For the analysis of filamentous fungi, grown on solid

| Biochemical identity | MALDI-TOF Match | MALDI-TOF Mismatch | No MALDI-TOF Result |
|---|---|---|---|
| *Candida albicans* (1) | 0 | 0 | 1 |
| *Enterococcus faecalis* (1) | 1 | 0 | 0 |
| *Escherichia coli* (4) | 4 | 0 | 0 |
| *Lactobacillus paracasei* (1) | 0 | 0 | 1 |
| *Neisseria meningitidis* (1) | 1 | 0 | 0 |
| *Propionibacterium acnes* (1) | 0 | 0 | 1 |
| *Pseudomonas aeruginosa* (1) | 1 | 0 | 0 |
| *Staphylococcus aureus* (1) | 1 | 0 | 0 |
| *Staphylococcus epidermidis* (8) | 8 | 0 | 0 |
| *Staphylococcus haemolyticus* (1) | 1 | 0 | 0 |
| *Staphylococcus hominis* (1) | 1 | 0 | 0 |
| *Streptococcus mitis* (1) | 0 | 1 [1] | 0 |
| *Streptococcus pneumoniae* (1) | 1 | 0 | 0 |
| ∑ (23) | 19 | 1 | 3 |

[1] MALDI-TOF MS: *Streptococcus pneumoniae*

**Table 1:** Results for the identification of microorganisms from positive blood culture broths analyzed by MALDI-TOF MS compared to traditional methods.

culture media, the sample preparation was carried out using the ethanol/formic acid-extraction procedure (protocol 2). However, it must be ensured that the pellet is dried completely before adding the 30 μl of formic acid. This is important to prevent dilution of the acid and therefore ineffectiveness of this preparation step. Because of the sponge-like texture of filamentous fungi, the drying process may take up to 60 min.

However, many fungi grow deep inside the solid agar, thus complicating sample harvesting without agar contamination. Therefore, fungi were also incubated with agitation in Sabouraud bouillon. After 3-10 days (pathogen specific), media, containing fungi, was transferred into a micro tube and centrifuged for 2 min at 12,500 g. The supernatant was discarded and the pellet resuspended in 1 ml deionized water, followed by a further centrifugation step used to remove any remaining media. After discarding the supernatant, the preparation continued with protocol 2.

**Protocol 4:** The sample preparation from positive blood culture broths represents a special case. The microorganisms were detected directly without further cultivation. Therefore, an additional isolation step has to be applied prior to analysis (Figure 3). After mixing the positive blood culture bottle, 8 ml blood were transferred to a BD Vacutainer SST II Advance tube (Becton Dickinson, Heidelberg, Germany) and centrifuged for 10 min at 2,000 g. After the supernatant (serum) was removed carefully, the bacterial pellet, located on the gel, was resuspended in 300 μl deionized water and transferred to a micro tube. A second centrifugation step was applied followed by the formic acid-extraction procedure described before (Protocol 2).

### MS parameters and result management

Spectra were recorded using a microflex LT mass spectrometer (Bruker Daltonics, Bremen, Germany) in linear mode equipped with a pulsed $N_2$ laser ($\lambda$=337 nm). Mass spectra were acquired over an m/z range from 2,000 to 20,000. The acceleration voltage was 20,000 V. After spectrum acquisition data was processed automatically by the analysis software Biotyper® v.2.0 (Bruker Daltonics). The cut off score values for reliable species identification, proposed by the manufacturer [16], were used for all samples grown on solid media and fungi grown in Sabouraud bouillon. However, for identification of blood culture samples the cut-off, indicating reliable identification, was set to ≥ 1.7.

MS data was routinely compared to standardized microbiological methods. Matching results were considered positive and registered accordingly. Non-concordant results were double-checked at all levels. Additional data were collected using the automated Phoenix system

(BD, Heidelberg, Germany) that is based on standard biochemistry combined with fluorogenically and chromogenically modified substrates. Data derived from sequencing 16S rDNA [19] and NCBI data base researches were also included into final species determination.

## Results

Initially, bacteria (*E. coli*, *S. aureus*) were cultured for various intervals (5 h, 18 h, 24 h, 48 h) and on diverse solid agars. Although few peaks altered intensity or were missed under certain conditions, variation of growth conditions did not interfere with authentic species identification. As demonstrated in Figure 2, score values remained constant under various conditions.

Similar score values were obtained when fungi were grown either in liquid cultures or on agar plates (Table 2). Culture of dermatophytes and molds in liquid media resulted in faster species identification than culture on solid agar plates. Furthermore, growth in liquid media facilitated generation of a clean pellet for sample preparation.

In this study, 192 different bacterial and 42 different fungal species (15 yeasts and 27 molds/dermatophytes), were examined. Almost all MALDI-TOF MS results were confirmed directly by standard microbiological testing - predominantly by using the API identification system for bacteria and yeast, and standard morphological identification procedures for molds and dermatophytes (98%; 2,831/2,900) (Figure 4).

Beside bacteria and yeasts, all (except four strains; due to database error) dermatophytes and molds were reliably identified. 59 of the 63 samples gave concordant identification results. Even when score values < 1.7 were obtained, species identification matched the reference methods. Furthermore, MALDI-TOF MS allowed very rapid identification of molds and dermatophytes, i.e., these isolates were identified after cultivation in liquid media after 3-10 days, whereas results from fungi grown on solid media were obtained after 5-14 days.

Additionally, 23 clinical samples of positive blood cultures (directly after microbial growth was registered) were analyzed (Table1). Of these, 19 samples were identified correctly when compared to the traditional
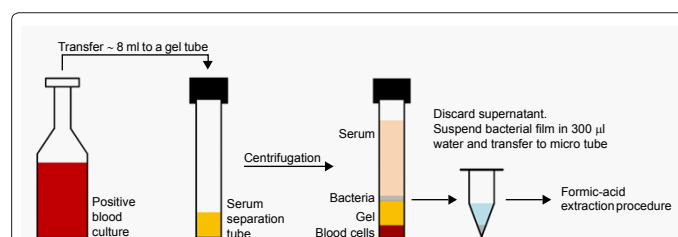


**Figure 3:** Workflow of sample preparation for the identification of microorganisms directly from positive blood cultures. 8 ml blood are transferred to a serum separation tube and centrifuged for 10 min at 2,000 g. The supernatant (serum) is removed carefully. Then the bacterial pellet, located on the gel, is resuspended in 300 μl deionized water and transferred to a micro tube. A second centrifugation step is applied followed by the formic acid extraction procedure and the preparation on the MALDI target plate.

| Score value | Solid media | Liquid media |
|---|---|---|
| No spectrum | 0 | 1 |
| 0.000 − 1.699 | 7 | 12 |
| 1.700 − 1.999 | 32 | 25 |
| 2.000 − 2.299 | 8 | 3 |
| > 2.3 | 12 | 12 |
| ∑ | 59 | 54 [1] |

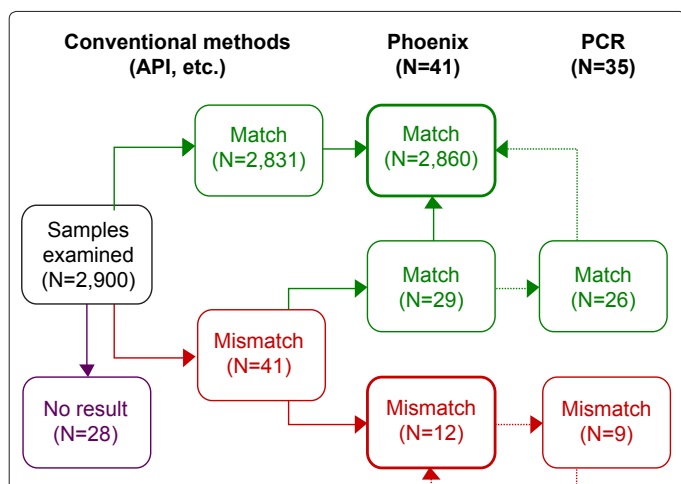[1] 5 samples were solely taken from solid media.

**Table 2:** Distribution of score values received from MALDI-TOF MS analyses of dermatophyte and mold samples with regard to cultivation conditions.

methods. Three samples (2 anaerobic bacteria, 1 yeast) showed no reliable results (score value < 1.3) when analyzed by MALDI-TOF MS. For one sample, an incongruent result was received. The incorrect identification by MALDI-TOF MS, however, was due to database discrepancies. Thus, 82% of examined blood culture samples were identified correctly within 30 min after microbial growth was registered in the bottles.

Regarding all 2,900 strains analyzed in this study, 41 (Table 3) MALDI-TOF MS based species identifications were discrepant to results obtained by conventional methods (e.g. API system). Among these discrepant results, MALDI-TOF identification was confirmed by sequence analysis in 26 cases and in three additional cases by the Phoenix system. In 12 of the discrepant cases MALDI-TOF analysis did not result in correct species identification (Table 4). In nine of these cases, Phoenix and sequencing results were in agreement but were discrepant to MALDI-TOF MS analysis; in three discrepant cases DNA sequencing was not available.

For 28 additional isolates, it was not possible to compare MALDI-TOF results to identification results obtained with other methods. Accordingly, these isolates were excluded from comparison. Nine of these strains belonged to species, which were not listed in the database: *Candida pelliculosa, C. nivariensis, C. ethanolytica, Moraxella phenylpyruvicus, Nocardia veterano, Ochrobactrum antropi, Dermacoccus nishinomiyaens, Wautersia paucula, Tsukamurella tyrosinosolvens*. Therefore, no reliable identification was received. In 15 further cases (*Aerococcus urinae, Arthrobacter cuminsii, Helicobacter pylori* (n=8), *Nocardia farcinica, Trichophyton interdigitale* (n=4)), unreliable identifications resulted from contaminations by other bacteria or also from database errors. In four additional cases, species assignment remained doubtful because all methods showed contrasting results (sample confusion most probable).

In summary, 2,860 of 2,900 (99%) samples identified by MALDI-TOF MS matched the identification results obtained with other methods (standard and high end microbiological identification methods including automated biochemical analyses and molecular identification).

| MALDI-TOF MS Results | Conventional Methods (N=2,872)[1] | | Phoenix (N=41) | | PCR (N=35) | |
|---|---|---|---|---|---|---|
| | Match | Mismatch | Match | Mismatch | Match | Mismatch |
| Enterobacteriaceae (n=1081) | 1064 | 17 | 14 | 3 | 14 | 3 |
| Micrococcaceae (n=407) | 405 | 2 | 2 | 0 | 2 | 0 |
| Streptococcaceae (n=448) | 442 | 6 | 1 | 5 | 0 | 3 |
| Nonfermenters (n=288) | 280 | 8 | 6 | 2 | 5 | 1 |
| Bacteria anaerobe (n=156) | 155 | 1 | 0 | 1 | 0 | 1 |
| Gram positive rods aerob (n=89) | 86 | 3 | 3 | 0 | 3 | 0 |
| Miscellaneous (n=163) | 159 | 4 | 3 | 1 | 2 | 1 |
| Fungi (n=240) | 240 | 0 | 0 | 0 | 0 | 0 |
| Total (n=2872)[1] | 2831 | 41 | 29 | 12 | 26 | 9 |

[1] 28 additional isolates were excluded from the comparison, because it was not possible to compare their MALDI-TOF results to identification results obtained with other methods.

**Table 3:** Comparison of MALDI-TOF MS species identification with species identification by other methods. The results are summarized in accordance to various groups of microorganisms.

| Serial number | MALDI-TOF MS | Phoenix / PCR |
|---|---|---|
| 1 | *Streptococcus pneumoniae* | *Streptococcus anginosus* |
| 2 | *Streptococcus pneumoniae* | *Streptococcus mitis* |
| 3 | *Streptococcus pneumoniae* | *Streptococcus mitis* |
| 4 | *Streptococcus pneumoniae* | *Streptococcus mitis* |
| 5 | *Streptococcus pneumoniae* | *Streptococcus mitis* |
| 6 | *Raoultella ornithinolytica* | *Klebsiella oxytoca* |
| 7 | *Raoultella ornithinolytica* | *Klebsiella oxytoca* |
| 8 | *Raoultella ornithinolytica* | *Klebsiella oxytoca* |
| 9 | *Acinetobacter ursingii* | *Acinetobacter lwoffii* |
| 10 | *Bordetella bronchiseptica* | *Bordetella parapertussis* |
| 11 | *Haemophilus influenzae* | *Pasteurella multocida* |
| 12 | *Eubacterium brachy* | *Propionibacterium acnes* |

**Table 4:** Species of bacterial strains obviously misidentified by MALDI-TOF MS.

## Discussion

In the present study, we describe the implementation of MALDI-TOF MS for species identification of microorganisms cultured from various routine samples (swabs, respiratory secretions, urine, blood culture, etc.). The results of this study show that MALDI-TOF MS is a robust method to identify a vast majority of bacterial and fungal species and also allows direct identification of microorganisms directly from positive blood cultures.

In total, 2,860 from 2,900 (99%) MALDI-TOF MS results were confirmed by other methods. This conformity is in the range as observed by others [20,21].

Although identification of bacteria worked very well by MALDI-TOF MS overall, there were a few difficulties. One problem was the lack of reference spectra in the database, resulting in 9 cases which could not be allocated to the respective species. However, the present study was performed in the year 2009, and in between, databases were extended suggesting that many of these isolates would be identified correctly nowadays. This assumption is confirmed by the fact that recently these species were identified correctly in other laboratories after database updates [22].

Another diagnostic problem is the differentiation of *S. mitis* group bacteria and *S. pneumoniae*. This problem has been observed previously by others [23,24]. Interestingly, 16S rRNA gene sequence-based identification also suffers from this limitation [25] indicating

**Figure 4:** Validation of MALDI-TOF MS results in microbiological diagnostics. 2,900 samples were examined by methods used for routine analysis (e.g. API system) and by MALDI-TOF MS. Mismatching results were subsequently examined by the Phoenix system (41 strains) and by 16S rDNA gene sequencing (35 of 41 strains). For 28 samples no feasible results were obtained by MALDI-TOF MS analysis.

that difficulties may rely on high homology of ribosomal proteins. In another study MALDI-TOF MS was not able to distinguish closely related viridans group streptococci [26] confirming the idea that high homology of ribosomal proteins in viridans streptococci impede species identification by MALDI-TOF MS. However, Dunne et al. [27] showed that MALDI-TOF MS analysis of multilocus sequence typing fragments matched those obtained by traditional sequence-based MLST for 99% of alleles. This finding indicates that MALDI-TOF MS might be a useful tool for epidemiologic studies of pneumococci. On the other hand, misidentification of pneumococci and *S. mitis* may have clinical consequences when disregarded [28].

In three cases, MALDI-TOF MS resulted in *Raoultella ornithinolytica* whereas Phoenix/sequence analyses identified *Klebsiella oxytoca*. Previously, *R. ornithinolytica* was named *Klebsiella ornithinolytica*. Therefore, this mismatch concerns two closely related species.

Although MALDI-TOF MS resulted in a few misidentifications, in general this method provided reliable results. Even when culturing bacteria for various periods and on different agar types the species were identified correctly with score values > 2.3 indicating that analysis result was reliable. Therefore, MALDI-TOF MS is a very robust method even when bacteria are grown on various solid media. This observation partially contrasts to the finding of a recent study [29] in which various bacterial species were cultured on a variety of agar types. While many species were identified in consisting quality after culture of various agar types, other species score values decreased after culture on certain agar plates. The authors found that this effect was most prominent for staphylococci cultured on CNA and for *Pseudomonas spp.* cultured on MacConkey agar. MacConkey agar contains crystal violet, a substance which possibly interferes with mass peak signals of the analytes [30].

Although the spectrum depends on the culture period, score values were not influenced by the growth time. However, a period of less than 5 hours might result in an insufficient amount of sample material. This observation has also been described previously [30].

In the present study, MALDI-TOF MS results were reliable not only for the identification of bacteria but also for fungi. As already described, MALDI-TOF MS is an appropriate tool to identify yeasts [30–32]. In contrast to bacteria and yeasts, protein extraction was routinely performed on molds (filamentous fungi) and dermatophytes. Performing protein extraction allowed solid species identification. This finding agrees with that of De Carolis et al. [33], describing MALDI-TOF MS an appropriate tool for routine identification of filamentous fungi in a medical microbiology laboratory. However, for routine analysis it is of concern that the pigment of darkly pigmented fungi impairs MALDI-TOF MS analysis [34]. Interestingly, this phenomenon was not observed when analysing non-pigmented parts of the same mold strain indicating that MALDI-TOF MS analysis should be performed on non-pigmented sample material. Fortunately, MALDI-TOF MS analysis was also adequate for species identification of dermatophytes [17,35,36].

To accelerate identification of dermatophytes and molds, these fungi were also grown in liquid cultures. While morphological species identification might last up to six weeks, MALDI-TOF MS based species identification could trustworthy be performed after culture periods of 3-10 days. Similarly in a recent report, De Respinis et al. [17] analyzed dermatophytes by MALDI-TOF MS after 3 days of incubation, using young, growing mycelium. Beside appropriate species identification, MALDI-TOF MS analysis allows rapid identification of fungi that otherwise must be cultured for longer periods. As some filamentous

fungi intrinsically are not susceptible to antifungal drugs, early knowledge of fungal species might contribute to appropriate treatment regime.

In recent years, direct identification from positive blood cultures by MALDI-TOF MS has emerged as a new approach to advance the identification process [37–41]. Blood cultures are primarily sterile samples, which means, most pathogens causing a bloodstream infection are present as pure cultures. This is an important prerequisite for the direct analysis of such samples by MALDI-TOF MS. Different methods for the preparation of blood culture broths prior to MS analysis have been developed [23,24,37].

In this study, a modified extraction procedure, based on a protocol described by Groebner et al. [42], for the direct analysis of staphylococci from positive blood cultures by real-time PCR, was applied. The results, however, yielded lower score values than the results obtained after additional cultivation. One reason for this is the low concentration of pathogens in blood culture broths (directly when detected positive), which results in lower signal response. Additionally, the incomplete separations of pathogens from broth and blood components, whose proteins interfere with the sample, disrupt the mass spectrum. Thereby database comparison is hampered and poor score values are received. However, in most cases the results still provide score values which indicate a reliable identification. In this particular case the cut off for score values indicating a reliable identification was set to ≥ 1.7. The isolation and identification of anaerobic bacteria with the used procedure seems to lead to unsatisfactory results. A precise statement about the suitability of this method for such pathogens cannot be made, considering the low number of examined anaerobic blood culture samples. Nevertheless, the majority of tested positive blood culture samples (82%) were identified correctly. The pathogens were identified within 30 minutes (after initial growth in the blood culture broths). MALDI-TOF MS for rapid identification of microorganisms from blood cultures can reduce the turnaround time to identification and may lead to earlier appropriate treatment. This shows the enormous potential of the MALDI-TOF MS for such an important clinical application. Meanwhile, in the Synlab laboratory Weiden bacterial analysis from blood cultures bottles has been implemented to obtain preliminary species identification while bacteria subsequently grown on agar plates are used for final species identification.

In summary, our study shows that species identification by MALDI-TOF MS facilitates reliable and rapid identification of microorganisms grown on solid media and in liquid media. A noticeable acceleration of the identification process was obtained for blood cultures and for dermatophytes and molds cultured in liquid media. MALDI-TOF MS has proven a promising alternative for traditional identification methods, and is on its way to become a new standard for the identification of microorganisms in high throughput laboratories.

## Perspectives and outlook

At least in developed countries MALDI-TOF MS technology is on the rise. In Germany, most of the large commercial laboratories use this technology since in high throughput laboratories investment costs are rapidly more than compensated by economies due to decreased usage of consumable material. In the past years, efforts have been made to extend the usage of MALDI-TOF MS to further applications. One application is the identification of designated subgroups of a certain species, e.g. to differentiate between Methicillin susceptible and Methicillin resistant *S. aureus* (MRSA) [10]. Although studies performed so far failed to

distinguish between MRSA and methicillin susceptible *S. aureus*, it was possible to generate *S. aureus* spectra allowing strain to strain differentiation [43]. Furthermore, discriminatory power of MALDI-TOF MS was similar when compared to single nucleotide polymorphism (SNP) plus binary gene analysis typing to detect inter-strain relatedness of *S. aureus* [44]. MALDI-TOF MS might also be a usable tool to type other species than *S. aureus*. Recently, it was possible to differentiate *Salmonella enterica* serovar typhi from other serovars [45]. In another study, the five most common serovars of *S. enterica* were identified by using a sophisticated algorithm for interpretation of obtained spectra [46]. When pneumococci strains had been typed by MADI-TOF MS and by multi-locus sequence-typing (MLST), 99% of obtained results from both methods matched [27]. Reil et al. [47] described a procedure to identify *C. difficile* strains 027, 001 and 126/078 by MALDI-TOF MS. Therefore, MALDI-TOF MS based analyses of bacterial strains at subspecies level might substitute established but elaborated techniques in future.

Another topic where MALDI-TOF MS could be applied is the identification of antibiotic susceptibility profiles. Some studies have demonstrated bacterial resistance by verifying degradation products of certain antibiotics [48–50]. Other studies showed that certain spectrum peaks were associated with presence or absence of antibiotic degrading enzymes allowing assessment of susceptibility to a certain antibiotic [51,52]. However, protocols to obtain comprehensive resistance patterns are not available these days.

A further application of MALDI-TOF MS might be to detect the presence of pathogenicity factors. For instance a peak occurring at m/z 4,448 when analyzing *S. aureus* strains had been identified to indicate presence of Panton Valentin Leucocidin (PVL) [53]. However, the results of a recent study challenge this relation [54].

In summary, MALDI-TOF MS is an appropriate tool to accelerate and to improve the quality of traditional microbiological diagnostics.

## References

1. Beuhler RJ, Flanigan E, Greene LJ, Friedman L (1974) Proton transfer mass spectrometry of peptides. A rapid heating technique for underivatized peptides containing arginine. J Am Chem Soc 96: 3990–3999.

2. Anhalt JP, Fenselau C (1975) Identification of bacteria using mass spectrometry. Anal Chem 47: 219–225.

3. Hillenkamp F, Karas M (1990) Mass spectrometry of peptides and proteins by matrix-assisted ultraviolet laser desorption/ionization. Methods Enzymol 193: 280–295.

4. Claydon MA, Davey SN, Edwards-Jones V, Gordon DB (1996) The rapid identification of intact microorganisms using mass spectrometry. Nat Biotechnol 14: 1584–1586.

5. Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, et al. (1996) Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10: 1227–1232.

6. Demirev PA, Ho YP, Ryzhov V, Fenselau C (1999) Microorganism identification by mass spectrometry and protein database searches. Anal Chem 71: 2732–2738.

7. Fenselau C, Demirev PA (2001) Characterization of intact microorganisms by MALDI mass spectrometry. Mass Spectrom Rev 20: 157–171.

8. Wilkins CL, Lay JO (2006) Identification of microorganisms by mass spectrometry. John Wiley & Sons, Hoboken.

9. Hillenkamp F, Peter-Katalinic J (2007) MALDI MS A practical guide to instrumentation, methods and applications Wiley-VCH, Weinheim.

10. Carbonnelle E, Mesquita C, Bille E, Day N, Dauphin B, et al. (2011) MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory. Clin Biochem 44: 104–109.

11. Karas M, Glückmann M, Schäfer J (2000) Ionization in matrix-assisted laser desorption/ionization: singly charged molecular ions are the lucky survivors. J Mass Spectrom 35 : 1–12.

12. Jaskolla TW, Karas M (2011) Compelling evidence for Lucky Survivor and gas phase protonation: the unified MALDI analyte protonation mechanism. J Am Soc Mass Spectrom 22: 976–988.

13. Van Belkum A, Welker M, Erhard M, Chatellier S (2012) Biomedical mass spectrometry in today's and tomorrow's clinical microbiology laboratories. J Clin Microbiol 50: 1513–1517.

14. Jones JJ, Stump MJ, Fleming RC, Lay JO Jr, Wilkins CL (2003) Investigation of MALDI-TOF and FT-MS techniques for analysis of *Escherichia coli* whole cells. Anal Chem 75: 1340–1347.

15. Valentine N, Wunschel S, Wunschel D, Petersen C, Wahl K (2005) Effect of culture conditions on microorganism identification by matrix-assisted laser desorption ionization mass spectrometry. Appl Environ Microbiol 71: 58–64.

16. Bruker Daltonic (2008) MALDI Biotyper user manual, version 20 SR1.

17. De Respinis S, Tonolla M, Pranghofer S, Petrini L, Petrini O, et al. (2012) Identification of Dermatophytes by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Medical mycology In press.

18. Ciardo DE, Schär G, Altwegg M, Böttger EC, Bosshard PP (2007) Identification of moulds in the diagnostic laboratory--an algorithm implementing molecular and phenotypic methods. Diagn Microbiol Infect Dis 59: 49-60.

19. Goldenberger D, Künzli A, Vogt P, Zbinden R, Altwegg M (1997) Molecular diagnosis of bacterial endocarditis by broad-range PCR amplification and direct sequencing. J Clin Microbiol 35: 2733–2739.

20. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, et al. (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49: 543–551.

21. Benagli C, Rossi V, Dolina M, Tonolla M, Petrini O (2011) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the identification of clinically relevant bacteria. PLoS ONE 6: e16424.

22. Bizzini A, Jaton K, Romo D, Bille J, Prod'hom G, et al. (2011) Matrix-assisted laser desorption ionization-time of flight mass spectrometry as an alternative to 16S rRNA gene sequencing for identification of difficult-to-identify bacterial strains. J Clin Microbiol 49: 693–696.

23. Christner M, Rohde H, Wolters M, Sobottka I, Wegscheider K, et al. (2010) Rapid identification of bacteria from positive blood culture bottles by use of matrix-assisted laser desorption-ionization time of flight mass spectrometry fingerprinting. J Clin Microbiol 48: 1584–1591.

24. Ferroni A, Suarez S, Beretti JL, Dauphin B, Bille E, et al. (2010) Real-time identification of bacteria and Candida species in positive blood culture broths by matrix-assisted laser desorption-ionization-time of flight mass spectrometry. J Clin Microbiol 48: 1542–1548.

25. Doern CD, Burnham CA (2010) It's not easy being green: the viridans group *streptococci*, with a focus on pediatric clinical manifestations. J Clin Microbiol 48: 3829–3835.

26. López Roa P, Sánchez Carrillo C, Marín M, Romero F, Cercenado E, et al. (2012) Value of matrix-assisted laser desorption ionization-time of flight for routine identification of viridans group *streptococci* causing bloodstream infections. Clin Microbiol Infect. In press.

27. Dunne EM, Ong EK, Moser RJ, Siba PM, Phuanukoonnon S, et al. (2011) Multilocus sequence typing of Streptococcus pneumoniae by use of mass spectrometry. J Clin Microbiol 49: 3756–3760.

28. Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G (2010) Performance of matrix-assisted laser desorption ionization-time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol 48: 1549–1554.

29. Anderson NW, Buchan BW, Riebe KM, Parsons LN, Gnacinski S, et al. (2012) Effects of solid-medium type on routine identification of bacterial isolates by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 50: 1008–1013.

30. Croxatto A, Prod'hom G, Greub G (2012) Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiol Rev 36: 380–407.

31. Marklein G, Josten M, Klanke U, Müller E, Horré R, et al. (2009) Matrix-assisted laser desorption ionization-time of flight mass spectrometry for fast and reliable identification of clinical yeast isolates. J Clin Microbiol 47: 2912–2917.

32. Bader O, Weig M, Taverne-Ghadwal L, Lugert R, Gross U, et al. (2011) Improved clinical laboratory identification of human pathogenic yeasts by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect 17: 1359–1365.

33. De Carolis E, Posteraro B, Lass-Flörl C, Vella A, Florio AR, Torelli R, et al. (2012) Species identification of Aspergillus, Fusarium and Mucorales with direct surface analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Clin Microbiol Infect 18: 475–484.

34. Buskirk AD, Hettick JM, Chipinda I, Law BF, Siegel PD, et al. (2011) Fungal pigments inhibit the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of darkly pigmented fungi. Anal Biochem 411: 122–128.

35. Erhard M, Hipler UC, Burmester A, Brakhage AA, Wöstemeyer J (2008) Identification of dermatophyte species causing onychomycosis and tinea pedis by MALDI-TOF mass spectrometry. Exp Dermatol 17: 356–361.

36. Nenoff P, Erhard M, Simon JC, Muylowa GK, Herrmann J, et al. (2012) MALDI-TOF mass spectrometry - a rapid method for the identification of dermatophyte species. Med Mycol 51: 17-24.

37. Stevenson LG, Drake SK, Murray PR (2010) Rapid identification of bacteria in positive blood culture broths by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 48: 444–447.

38. Kok J, Thomas LC, Olma T, Chen SC, Iredell JR (2011) Identification of bacteria in blood culture broths using matrix-assisted laser desorption-ionization Sepsityper™ and time of flight mass spectrometry. PLoS ONE 6: e23285.

39. La Scola B (2011) Intact cell MALDI-TOF mass spectrometry-based approaches for the diagnosis of bloodstream infections. Expert Rev Mol Diagn 11: 287–298.

40. Vlek AL, Bonten MJ, Boel CH (2012) Direct Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry Improves Appropriateness of Antibiotic Treatment of Bacteremia. PLoS ONE 7: e32589.

41. Wüppenhorst N, Consoir C, Lörch D, Schneider C (2012) Direct identification of bacteria from charcoal-containing blood culture bottles using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. Eur J Clin Microbiol Infect Dis 31: 2843–2850.

42. Gröbner S, Kempf VA (2007) Rapid detection of methicillin-resistant staphylococci by real-time PCR directly from positive blood culture bottles. Eur J Clin Microbiol Infect Dis 26: 751–754.

43. Bernardo K, Pakulat N, Macht M, Krut O, Seifert H, et al. (2002) Identification and discrimination of Staphylococcus aureus strains using matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics 2: 747–753

44. Schlebusch S, Price GR, Hinds S, Nourse C, Schooneveldt JM, et al. (2010) First outbreak of PVL-positive nonmultiresistant MRSA in a neonatal ICU in Australia: comparison of MALDI-TOF and SNP-plus-binary gene typing. Eur J Clin Microbiol Infect Dis 29: 1311–1314.

45. Kuhns M, Zautner AE, Rabsch W, Zimmermann O, Weig M, et al. (2012) Rapid discrimination of Salmonella enterica serovar Typhi from other serovars by MALDI-TOF mass spectrometry. PLoS ONE 7: e40004.

46. Dieckmann R, Malorny B (2011) Rapid screening of epidemiologically important Salmonella enterica subsp. enterica serovars by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry.. Appl Environ Microbiol 77: 4136–4146.

47. Reil M, Erhard M, Kuijper EJ, Kist M, Zaiss H, et al. (2011) Recognition of Clostridium difficile PCR-ribotypes 001, 027 and 126/078 using an extended MALDI-TOF MS system. Eur J Clin Microbiol Infect Dis 30: 1431–1436.

48. Burckhardt I, Zimmermann S (2011) Using matrix-assisted laser desorption ionization-time of flight mass spectrometry to detect carbapenem resistance within 1 to 2.5 hours. J Clin Microbiol 49: 3321–3324

49. Hrabák J, Walková R, Studentová V, Chudáčková E, Bergerová T (2011) Carbapenemase activity detection by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49: 3222–3227.

50. Hooff GP, van Kampen JJ, Meesters RJ, van Belkum A, Goessens WH, et al. (2012) Characterization of β-lactamase enzyme activity in bacterial lysates using MALDI-mass spectrometry. J Proteome Res 11: 79–84.

51. Nagy E, Becker S, Sóki J, Urbán E, Kostrzewa M (2011) Differentiation of division I (cfiA-negative) and division II (cfiA-positive) Bacteroides fragilis strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Med Microbiol 60: 1584–1590.

52. Wybo I, De Bel A, Soetens O, Echahidi F, Vandoorslaer K, et al. (2011) Differentiation of cfiA-negative and cfiA-positive Bacteroides fragilis isolates by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 49: 1961–1964.

53. Bittar F, Ouchenane Z, Smati F, Raoult D, Rolain JM (2009) MALDI-TOF-MS for rapid detection of staphylococcal Panton-Valentine leukocidin. Int J Antimicrob Agents 34: 467–470.

54. Szabados F, Becker K, von Eiff C, Kaase M, Gatermann S (2011) The matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS)-based protein peaks of 4448 and 5302 Da are not associated with the presence of Panton-Valentine leukocidin. Int J Med Microbiol 301: 58–63.