# Rapid method for direct identification of bacteria in urine and blood culture samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: intact cell vs. extraction method

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## Abstract

Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is a fast and reliable technology for the identification of microorganisms with proteomics approaches. Here, we compare an intact cell method and a protein extraction method before application on the MALDI plate for the direct identification of microorganisms in both urine and blood culture samples from clinical microbiology laboratories. The results show that the intact cell method provides excellent results for urine and is a good initial method for blood cultures. The extraction method complements the intact cell method, improving microorganism identification from blood culture. Thus, we consider that MALDI-TOF MS performed directly on urine and blood culture samples, with the protocols that we propose, is a suitable technique for microorganism identification, as compared with the routine methods used in the clinical microbiology laboratory.

**Keywords:** Blood culture, clinical microbiology, identification, MALDI-TOF MS, urine
**Original Submission:** 20 April 2010; **Revised Submission:** 15 July 2010; **Accepted:** 16 July 2010
Editor: D. Raoult
**Article published online:** 12 August 2010
*Clin Microbiol Infect* 2011; **17:** 1007–1012
10.1111/j.1469-0691.2010.03339.x

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## Introduction

The identification of microorganisms in clinical microbiology laboratories is currently based on Gram staining, culture and growth characteristics, and biochemical patterns. These procedures are cumbersome and time-consuming. Molecular methods have been introduced recently, but they are expensive and time-consuming, and they are not currently suitable for routine identification. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is becoming a reliable method for the identification of microorganisms [1,2]. The use of MALDI-TOF MS for microorganism identification in clinical samples has been rare until recently, mainly because broad microorganism protein databases have become available only recently, and because of the high price of mass spectrometers [3–8]. Although universal sample preparation methods have been proposed [9], many different protocols are being used. The 'intact' cell technique, in which a single colony from a culture plate is spread onto the MALDI plate and overlaid with matrix, initially described in 1996 [2], has been applied for routine identification of bacteria in clinical samples [8,10]. Likewise, several methods for sample preparation and protein extraction have been proposed [11–13]. However, these approaches require culture of the original clinical specimen. Thus, the identification is delayed for at least 18–24 h, until visible colonies have grown, and can even be delayed for weeks for microorganisms such as *Mycobacterium tuberculosis*. Other clinically relevant bacteria, such as *Treponema pallidum*, simply fail to grow on culture media. Recently, several laboratories have described the identification of microorganisms directly from blood cultures (BCs) [14–20] and from urine [21] with different protein extraction methods (PEMs).

The aim of the present study was to compare the direct deposition of microorganisms onto the MALDI plate (intact cell method (ICM)) with the protein extraction and application on the MALDI plate for direct identification of clinical

microorganisms in urine and BC samples by MALDI-TOF MS.

## Materials and Methods

### Clinical samples

We analysed 238 urine samples and 68 BC samples submitted to the Microbiology Laboratory from inpatients and outpatients. Urine samples were collected in Vacutainer tubes without preservatives (Becton Dickinson, Franklin Lake, NJ, USA). Samples reported to be positive by the automated screening urine flow cytometry analyser (UF-1000i; Bio-Mérieux, Marcy L'Étoile, France) were cultured on blood agar and McConkey agar plates. Plates were incubated in an aerobic atmosphere at 37°C for 18 h. The colonies grown were identified by conventional methods. Only samples with colony counts $>10^5$ CFU/mL were considered for the study. MALDI-TOF MS was always performed within 24 h after sample collection, samples being kept at 4°C until their analysis.

Eight millilitres of blood, obtained aseptically by vein puncture, were inoculated into BC bottles (Becton Dickinson) and incubated at 37°C in a BD Bactec 9000 analyser (Becton Dickinson) until they were reported as positive or for a maximum of 10 days. No specific anaerobic bottles were used. Samples not reported as positive by the analyser after 10 days of incubation were discarded and reported as negative without plate culture. For samples reported by the analyser as presumptively positive, a Gram stain was performed, and 100 $\mu$L of the sample was cultured on blood, chocolate, McConkey and Sabouraud agar plates and incubated in an aerobic atmosphere with 10% $CO_2$ at 37°C for 18–24 h. Negative plates were incubated for a further 48 h, and if they remained negative they were discarded. When bacterial growth occurred, colonies were identified by conventional methods (WIDER MIC/ID Gram positive and WIDER MIC/ID Gram negative (MicroScan, Sacramento, CA, USA) and Auxacolor 2 (Bio-Rad, Marnes-la-Coquette, France)).

### MALDI-TOF MS

For MS, 4 mL of urine or BC were centrifuged at 2000 ***g*** for 30 s to remove blood cells. The supernatant was centrifuged at 15 500 ***g*** for 5 min to collect bacteria. The pellet obtained was washed once with de-ionized water. A small amount of sediment was spotted twice onto a target MALDI plate with a sterilized pipette tip, and left to dry in air at room temperature. This was the ICM (Fig. 1).

In the PEM, an ethanol–formic acid extraction method was used. The bacterial pellet was resuspended in 300 $\mu$L of water. Then, 900 $\mu$L of absolute ethanol was added, the mixture was centrifuged at 15 500 ***g*** for 2 min, and the supernatant was discarded. Fifty microlitres of formic acid (70% v/v) was added to the pellet and thoroughly mixed by pipetting before the addition of 50 $\mu$L of acetonitrile to the mixture. The mixture was centrifuged again at 15 500 ***g*** for 2 min. One microlitre of the supernatant was placed onto a spot of the steel target plate and air-dried at room temperature (Fig. 1).

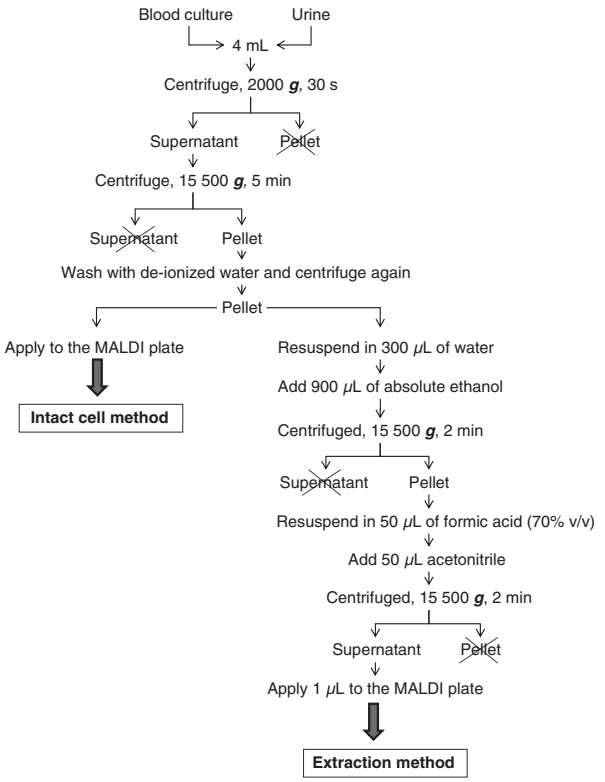


**FIG. 1.** Diagram of the general workflow in the analysis of urine and blood culture samples by matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry.

Both the microbial film and the supernatant of the extracted proteins were overlaid with 1 $\mu$L of matrix solution (a saturated solution of $\alpha$-cyano-4-hydroxycinnamic acid in organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid)) and air-dried.

MALDI-TOF MS was performed on an Autoflex III instrument (Bruker Daltonics, Leipzig, Germany) equipped with a 200-Hz smartbeam laser. The spectra were recorded in the positive linear mode at the maximum laser frequency within a mass range from 2000 to 20 000 Da. The instrument parameter settings were as follows: ion source 1 at 20 kV, ion source 2 at 18.6 kV, lens at 6 kV and an extraction delay time of 4 ns. For each spectrum, 500 laser shots were collected and analysed (50 laser shots from different positions of the target spot, ten times). The spectra were calibrated

externally with the standard calibration mixture (*Escherichia coli* extracts including the additional proteins RNase A and myoglobin; Bruker Daltonics). Calibration masses were as follows: RL36, 4364.3 Da; RS22, 5095.8 Da; RL34, 5380.4 Da; RLmeth, 6254.4 Da; RL32, 6315.2 Da; RL29, 7273.5 Da; RS19, 10229.1 Da; RNase A, 13682.2 Da; myoglobin, 16952.5 Da.

Spectra were analysed automatically with Biotyper 2.0 software (Bruker Daltonics), containing reference libraries of 3290 microbial species. The software performs normalization, smoothing, baseline subtraction and peak picking, creating a list of the most significant peaks of the spectrum (*m*/*z* values with a given intensity, with the threshold set to a minimum of 1% of the highest peak and a maximum of 100 peaks). To identify bacteria, the peak list generated was matched against reference libraries, with the integrated pattern-matching algorithm of the software. Once the spectrum was imported into the Biotyper software, the whole process, from processing to identification, was performed automatically. The results of the pattern-matching process were expressed as score values, which ranged from 0 to 3. MALDI-TOF MS identifications were classified by modifying score values proposed by the manufacturer: a score ≥2 indicated species identification, a score between 1.7 and 2 indicated genus identification, and a score <1.7 indicated no reliable identification.

## Results and Discussion

Two hundred and thirty-eight urine samples were processed directly by both plate culture and biochemical identification and MALDI-TOF MS. The microorganisms identified by MALDI-TOF MS are given in Table 1. Twenty urine samples reported as presumptively positive by the screening device were negative in culture. All of them were reported as 'no reliable identification' by MALDI-TOF MS. For the 218 urine samples confirmed to be positive by culture, conventional identification and MALDI-TOF MS were coincident at the species level in 203 cases, and at the genus level in 204 cases. MALDI-TOF MS failed to identify any microorganism in the 14 remaining samples, probably because the number of microorganisms in the sample was not high enough. We have reported that bacterial counts are critical to achieve reliable results in MALDI-TOF MS of clinical samples. When urine samples have a high bacterial load (>$10^5$ CFU/mL), correlation with conventional identification is 91.8% at the species level, and 92.7% at the genus level. Nevertheless, the number of microorganisms required for MALDI-TOF MS identification depends on the species. For example, in the case of *Enterococcus faecalis*, the bacterial count has to be higher than $5 \times 10^5$ CFU/mL [21].

**TABLE 1. Microorganism identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry in urine and blood culture samples**

| | Urine | Blood |
|---|---|---|
| **Microorganisms identified** | **No. of isolates** | **No. of isolates** |
| *Acinetobacter baumannii* | – | 1 |
| *Citrobacter freundii* | 1 | – |
| *Citrobacter koseri* | 1 | – |
| *Enterobacter asburiae* | 1 | – |
| *Enterobacter cloacae* | 6 | – |
| *Enterobacter hormaechei* | – | 1 |
| *Enterococcus faecalis* | 7 | 5 |
| *Escherichia coli* | 163 | 4 |
| *Klebsiella oxytoca* | 7 | – |
| *Klebsiella pneumoniae* | 7 | 3 |
| *Morganella morganii* | 1 | 1 |
| *Proteus mirabilis* | 4 | – |
| *Pseudomonas aeruginosa* | 1 | 2 |
| *Raoultella* sp. | 1 | – |
| *Serratia marcescens* | 2 | 2 |
| *Staphylococcus aureus* | 2 | 1 |
| *Staphylococcus epidermidis* | – | 15 |
| *Staphylococcus haemolyticus* | – | 2 |
| *Staphylococcus hominis* | – | 10 |
| *Staphylococcus* sp. | – | 12 |
| *Staphylococcus warneri* | – | 1 |
| *Streptococcus pneumoniae* | – | 2 |
| *Streptococcus pyogenes* | – | 3 |

We compared the results obtained with the ICM and the PEM for MALDI-TOF MS with the 20 urine samples negative in culture and with the 204 samples that gave the same results with conventional identification and MALDI-TOF MS (Fig. 2). All of the 20 culture-negative samples were reported as 'no reliable identification' by both the ICM and the PEM. Of the remaining 204 positive samples, 192 were coincident at the species level (94%), and 193 (95%) at the genus level. Fig. 3 shows that, for urine samples, spectra obtained for *Proteus mirabilis* and *Klebsiella pneumoniae* by both methods were very similar. We found some differences in only 11 positive samples (5%). Five samples (three *E. coli*, one *P. mirabilis* and one *Raoultella ornithinolytica*) were identified with the ICM (2%), but not with the PEM. Moreover, six samples (three *E. coli*, two *Enterococcus faecalis* and one *Enterobacter cloacae*) were only identified with the PEM (3%). The reason why some reliable identifications were obtained only with the ICM may be that, in some cases, most of the bacterial pellet was spread on the MALDI plate, and there might not have been enough of the remaining pellet for the subsequent PEM. The reason why some reliable identifications were obtained only with the PEM may be that this method increases cell wall lysis, enabling proteins to be thoroughly extracted, giving more and stronger signals.

In most samples, the score was >2 with both MALDI-TOF MS methods (170 of 204 cases with the ICM and 179 of 204 with the PEM). Scores between 1.7 and 2.0 were

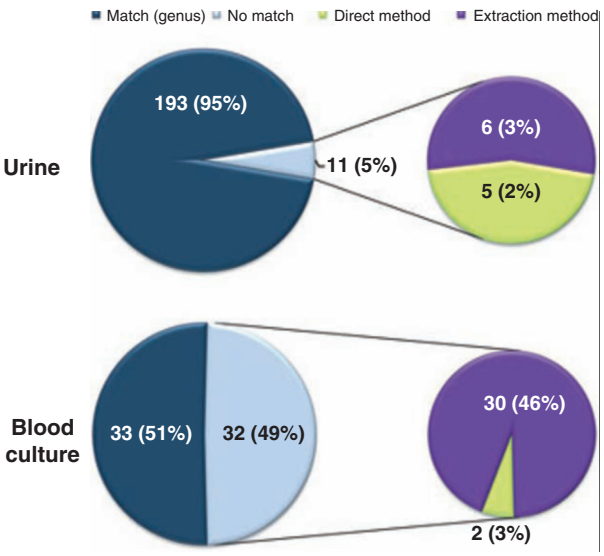


**FIG. 2.** Comparison of the intact cell method and the extraction method in urine and blood culture samples analysed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The samples compared are those with coincidence at the genus level with plate culture and biochemical identification.

obtained in 26 of 204 cases with the ICM and in 19 of 204 cases with the PEM. Scores <1.7 were obtained in eight cases with the ICM and in six cases with the PEM. The average scores were 2.20 for the ICM and 2.21 for the PEM. Among the cases coincident at species level, 100 samples had higher scores with the PEM and 90 samples had higher scores with the ICM. In two cases, the score was the same with both methods.

The MALDI-TOF MS identifications for BCs are shown in Table 1. When we compared the results obtained with conventional microbiological methods and MALDI-TOF MS, we found 76% (52/68 cases) coincident at the species level, and 96% (65/68) at the genus level. In 12 of 13 cases, the species identified only at the genus level were *Staphylococcus* species (two *Staphylococcus capitis*, eight *Staphylococcus epidermis*, one *Staphylococcus haemolyticus* and one *Staphylococcus hominis*); one of 13 cases was *Enterobacter*. We should keep in mind that, when MALDI-TOF MS and biochemical identification disagree, 16S rDNA sequencing frequently confirms MALDI-TOF MS results [19]. On the other hand, in three cases there was no identification by MALDI-TOF MS (4%), although plate culture and biochemical identification provided reliable identification (one *Staphylococcus aureus* and two *S. hominis*). Again, these MALDI-TOF MS identification failures are probably attributable to a low number of microorganisms in the sample. Christner *et al.* [17] have recently reported that, for microorganism identification in BCs by MALDI-TOF MS, microorganism concentrations >$10^6$ CFU/mL are needed to obtain suitable profiles for MS identification.

Concerning the methods compared, of the 65 samples identified by MALDI-TOF MS, 32 (49%) were correctly identified with both the ICM and the PEM at the species level, and 33 (51%) at the genus level. Thirty-two samples (49%) were correctly identified with only one method. Thirty samples (46%) were identified only with the PEM; the ICM gave 'no peaks found' or 'no reliable identification'. These samples were as follows: two *Staphylococcus warneri*, eight *S. hominis*, three *S. haemolyticus*, 10 *S. epidermidis*, one *S. aureus*, one *S. capitis*, two *Streptoccus pyogenes*, one *Streptoccus pneumoniae*, one *Serratia marcescens* and one *E. faecalis*. In two samples (one *S. epidermidis* and one *S. hominis*) (3%), correct identification was obtained only with the ICM, the PEM giving 'no peaks found' or 'no reliable identification' (Fig. 2).

Score values obtained for BC samples were lower than those for urine samples. Scores >2 were obtained in 18/65 BCs with the ICM, and in 35/65 with the PEM; scores were between 1.7 and 2.0 in 13 cases with the ICM, and in 18 cases with the PEM. Scores <1.7 were obtained in 34 cases with the ICM, and in 12 cases with the PEM. In most instances, even MALDI-TOF MS identifications with score values below 1.7 were coincident with conventional biochemical identification. The average scores were 1.75 for the ICM and 2.02 for the PEM. The PEM fundamentally and significantly improved the score in the genus *Staphylococcus*.

Routine identification procedures require at least 24–48 h. Recently, MALDI-TOF MS has been introduced in clinical laboratories, where rapid and reliable identification is of the utmost importance [3–8,14,15]. MALDI-TOF MS significantly reduces the time required for reliable identification, which can be obtained in minutes. Several studies have addressed the application of MALDI-TOF MS to the identification of microorganisms in BCs, and in all cases sample preparation included protein extraction procedures before spotting onto the target plate for MALDI-TOF MS measurements [14–20]. Nevertheless, there are no standardized protocols for sample preparation. La Scola and Raoult [14] used two different protocols, with a series of three centrifugation steps each, whereas Stevenson *et al.* [15] employed a protocol with a series of seven centrifugation steps. Ferroni *et al.* [18] used an extraction method with detergent and trifluoroacetic acid for extracting proteins. Christner *et al.* [17] processed bacterial cultures with formic acid extraction for MS. In all cases, the protocols are lengthy and require more processing steps and more sample manipulation than the method that we propose here. In our method, sample processing requires only urine and BC centrifugation, spreading of the sediment onto the MALDI plate, recording of the mass spectrum, and

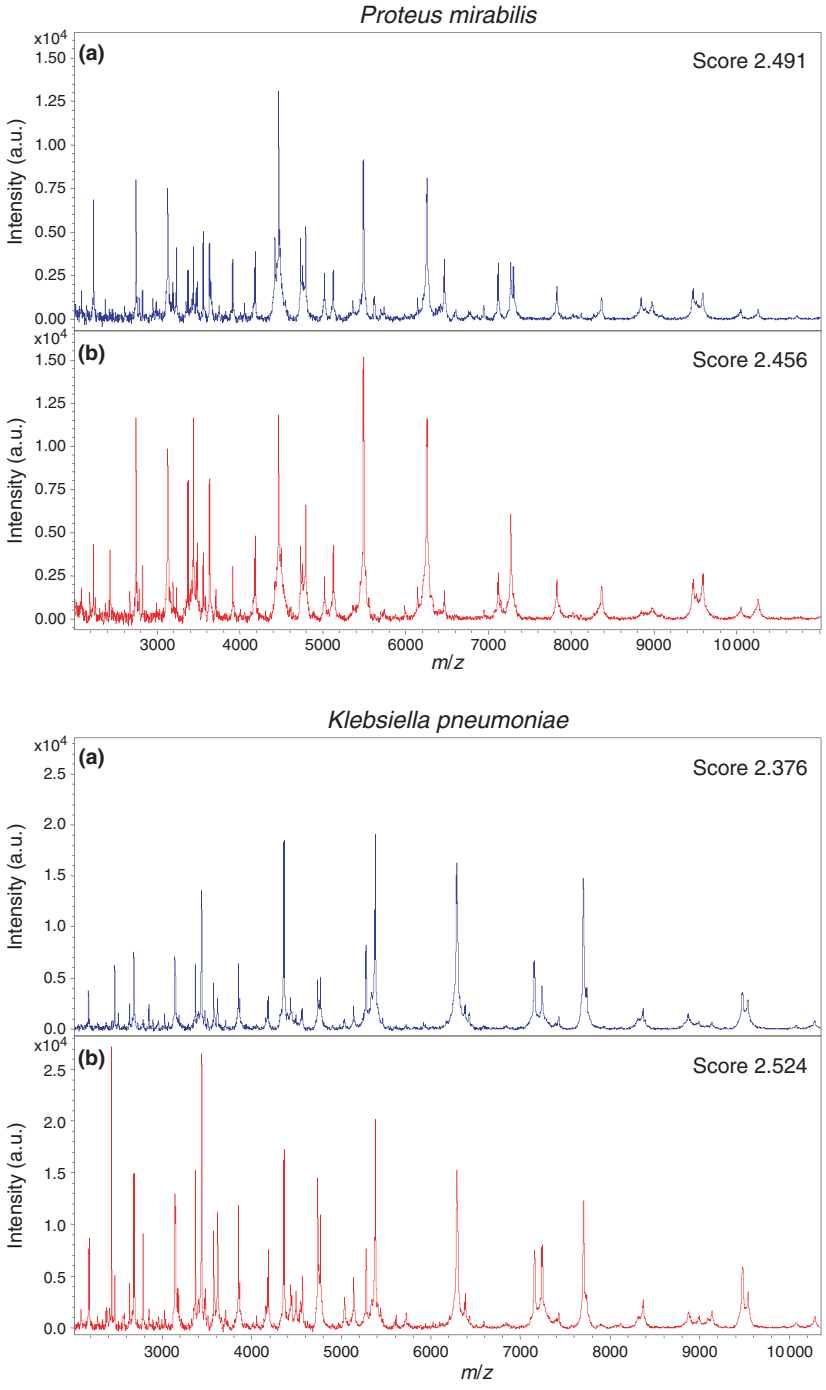


**FIG. 3.** Mass spectra (size 2.000–11.000 Da) of *Proteus mirabilis* and *Klebsiella pneumoniae* from urine samples obtained with the intact cell method (a) and with the protein extraction method (b). a.u., arbitrary units.

identification with the dedicated software for the ICM. The whole process takes about 10 min for one sample and about 20 min for 20 samples. The PEM imposes two more centrifugation steps, and the whole process takes about 20 min for one sample and 60 min for 20 samples.

Our results show that, with 4 mL of urine, the ICM gives almost the same results as the PEM, and is effective in 97% of cases. Therefore, it might not be necessary to extract the proteins before spreading the sample onto the MALDI plate, with the concomitant saving of time and the advantage of less sample manipulation. Regarding BCs, the PEM provides better results than the ICM, but identification with the ICM is reliable in half of the samples (49% at the species level and 51% at the genus level). We propose that the ICM should initially be applied to all samples and, if reliable identification is not achieved, the PEM should be applied to increase the number

of identifications. The protocol that we propose is easier and faster than that previously proposed for BCs [14–20].

In conclusion, MALDI-TOF MS is an outstanding method for the identification of microorganisms in clinical samples. The method is not time-consuming, and it is possible to report analytical results in minutes. The ICM affords excellent results with urine samples. Results for BC samples are not as significant as those for urine samples, but identification is reliable when it is obtained, and would reduce the number of samples to be processed with the PEM by 50%. In both cases, MALDI-TOF MS identification of microorganisms compares favourably with the routine methods currently used in clinical microbiology laboratories.

## Transparency Declaration

This work was partly supported by a grant from the Consejería de Sanidad, junta de Castilla y León and by the Instituto de Salud Carlos III (Ayuda de Infraestructura).

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