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Microorganisms direct identification from blood culture by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) allows a fast and reliable bacterial identification from culture plates. Direct analysis of clinical samples may increase its usefulness in samples in which a fast identification of microorganisms can guide empirical treatment, such as blood cultures (BC). Three hundred and thirty BC, reported as positive by the automated BC incubation device, were processed by conventional methods for BC processing, and by a fast method based on direct MALDI-TOF MS. Three hundred and eighteen of them yield growth on culture plates, and 12 were false positive. The MALDI-TOF MS-based method reported that no peaks were found, or the absence of a reliable identification profile, in all these false positive BC. No mixed cultures were found. Among these 318 BC, we isolated 61 Gram-negatives (GN), 239 Gram-positives (GP) and 18 fungi. Microorganism identifications in GN were coincident with conventional identification, at the species level, in 83.3% of BC and, at the genus level, in 96.6%. In GP, identifications were coincident with conventional identification in 31.8% of BC at the species level, and in 64.8% at the genus level. Fungaemia was not reliably detected by MALDI-TOF. In 18 BC positive for *Candida* species (eight *C. albicans*, nine *C. parapsilosis* and one *C. tropicalis*), no microorganisms were identified at the species level, and only one (5.6%) was detected at the genus level. The results of the present study show that this fast, MALDI-TOF MS-based method allows bacterial identification directly from presumptively positive BC in a short time (<30 min), with a high accuracy, especially when GN bacteria are involved.

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Introduction

Some 250 000 patients develop bacteraemia or fungaemia every year in the USA [1], and the trend towards an increasing incidence and a sustained, high mortality is apparent. Bloodstream infection pathogen (BSI)-associated mortality in the USA has been estimated to be 16–40% [2]. Early and appropriate empirical antimicrobial therapy is essential for survival of patients with sepsis [3–5]. Attributable mortality is approximately 20% for BSI patients treated with appropriate empirical therapy, and 34% for patients treated with inappropriate empirical therapy [3].

Methods allowing fast and reliable microorganism identification, directly from the positive blood culture (BC) bottle, would be decisive to guide empirical treatment. Several methods have been assayed to reduce the time to microbial species identification from the BC index [6–10].

Matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been suggested as a fast and reliable method for bacterial identification from plate cultures [11], based on protein profiles characteristic for each organism.

Direct analysis of clinical samples may further increase the usefulness of this method because it can shorten the time for identification of microorganism. In the present study, we

compared the conventional methods for BSI pathogen identification in microbiology laboratories and a fast method based on conventional detection of positive bottles plus MALDI-TOF MS directly on the BC.

Materials and Methods

Blood culture processing and biochemical identification

We analyzed 330 consecutive, nonselected BCs submitted to the microbiology laboratory, and reported as positive by the BC incubation device (BD Bactec 9000; Becton-Dickinson, Franklin Lajes, NJ, USA).

Eight millilitres of blood, obtained by vein puncture aseptically, were inoculated in BACTEC Plus + Aerobic/F bottles (Becton-Dickinson), and incubated at 37°C in a BACTEC 9240 device (Becton-Dickinson), until they were reported as positive by the incubation device, or for a maximum of 10 days. No specific anaerobic bottles were used. For samples reported by the BACTEC 9240 as presumptively positive, a Gram stain was performed, and 100 μL were cultured onto blood agar, chocolate, McConkey and Sabouraud agar plates. Plates were incubated in aerobic atmosphere with 10% CO_2, at 37°C for 18–24 h. Negative plates were incubated for an additional 48 h and, if they remained negative, were discarded. When there was colonies growth, microorganisms were identified by conventional methods [WIDER MIC/ID Gram-positives and WIDER MIC/ID Gram-negatives (manufactured for Francisco Soria Melguizo SA, Madrid (Spain) by MicroScan, Sacramento, CA, USA) for bacteria, and Auxacolor 2 (Bio-Rad, Marnes-la-Coquette, France) for fungi].

MALDI-TOF MS

Sample preparation for MALDI-TOF. Four millilitres of BC were centrifuged at 2000 ***g*** for 30 s to remove leucocytes. Supernatant was centrifuged at 15 500 ***g*** for 5 min to collect bacteria. The pellet was washed once with deionized water. Then, an ethanol/formic acid extraction procedure was applied: a small amount of bacteria was resuspended in 300 μL of water. Later, 900 μL of absolute ethanol was added and 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 mixed thoroughly before the addition of 50 μL of acetonitrile. 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 card and air-dried at room temperature.

The supernatant was overlaid with 1 μL of matrix solution [saturated solution of HCCA (α-cyano-4-hydroxy cinnamic acid) in organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid)] and air-dried.

MALDI-TOF MS. Measurements were performed in an Autoflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Leipzig, Germany) equipped with a 200-Hz smartbeam laser. Spectra were recorded in the linear, positive mode at a laser frequency of 200 Hz within a mass range from 2000–20 000 Da. The IS1 voltage was 20 kV, the IS2 voltage was maintained at 18.6 kV, lens voltage was 6 kV and the extraction delay time was 40 ns.

For each spectrum, 500 laser shots were collected and analyzed (10 × 50 laser shots from different positions of the target spot). The spectra were externally calibrated using the standard calibrant mixture (*Escherichia coli* extracts including the additional proteins RNase A and myoglobin; Bruker Daltonics). Calibration masses were: RL36, 4364.3 Da; RS22, 5095.8 Da; RL34, 5380.4 Da; RL33meth, 6254.4 Da, RL32, 6315 Da; RL29, 7273.5 Da; RS19, 10299.1 Da; RNase A, 13682.2 Da; myoglobin, 16952.5 Da.

Data analysis. For automated data analysis, raw spectra were processed using the MALDI BIOTYPER, version 2.0, software (Bruker Daltonics) with default settings. The software performs normalization, smoothing, baseline substraction 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 unknown bacteria, every peak list generated was matched directly against reference libraries (3290 species) using the integrated patterns matching algorithm of BIOTYPER, version 2.0 (Bruker Daltonics). The unknown spectra were compared with a library of reference spectra based on a pattern recognition algorithm using peak position, peak intensity distributions and peak frequencies.

Results scoring. MALDI-TOF identifications were classified using modified score values proposed by the manufacturer: a score ≥2 indicated species identification, a score in the range 1.7–1.9 indicated genus identification, and a score <1.7 indicated no identification.

Sequence data

When MALDI-TOF reported a reliable identification at the genus or species level, and it was different to conventional identification, this identification was confirmed by PCR for 16S RNA [12].

Results

Three hundred and thirty BCs reported as positive by the BACTEC 9240 device were processed both by plate culture

and conventional biochemical identification, and by MALDI-TOF MS. Twelve samples (3.6%) reported as positive by the BACTEC 9240 device were negative in culture. All of them were reported as revealing no reliable identification or no peaks found by the MALDI-TOF MS.

Three hundred and eighteen samples led to growth of one colony morphology in culture. No mixed cultures were found. The identification is shown in Table 1. MALDI-TOF MS and conventional methods reported the same identification for *Enterobacteriaceae*, at the species level, in 43 of 48 BC bottles (89.6%), and at the genus level in 47 of 48 bottles (97.9%). Nonfermentative Gram-negative bacilli were identified at the species level in eight of 12 bottles (66.7%), and at the genus level in 100%. As a whole, correlation in BCs

TABLE 1. Identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and conventional identification of 318 microorganisms isolated from blood cultures

Conventional identification (number of isolates)	Correlation at the species level (%)	Correlation at the genus level (%)	MALDI-TOF identification (number of isolates)
Acinetobacter baumanii (2)	50	100	*Acinetobacter baumanii* (1) *Acinetobacter* sp. (1)
Bacillus fusiformis (1)	–	100	*Bacillus* sp. (1)
Candida albicans (8)	–	–	No reliable ID (8)
Candida parapsilosis (9)	–	11.1	*Candida* sp. (1) No reliable ID (7) No peaks found (1)
Candida tropicalis (1)	–	–	No reliable ID (1)
Corynebacterium amycolatum (1)	–	–	No reliable ID (1)
Enterobacter amnigenus (1)	–	–	No reliable ID (1)
Enterobacter spp. (1)	–	100	*Enterobacter hormaechei* (1)
Enterococcus faecalis (14)	78.6	78.6	*Enterococcus faecalis* (11) No reliable ID (3)
Enterococcus faecium (3)	33.3	66.7	*Enterococcus faecium* (1) *Enterococcus* sp. (1) No reliable ID (1)
Escherichia coli (29)	96.5	100	*Escherichia coli* (28) *Escherichia* sp. (1)
Francisella tularensis (1)	–	–	No reliable ID (1)
Klebsiella oxytoca (4)	100	100	*Klebsiella oxytoca* (4)
Klebsiella pneumoniae (6)	83.3	100	*Klebsiella pneumoniae* (5) *Klebsiella oxytoca* (1)
Listeria monocytogenes (1)	100	100	*Listeria monocytogenes* (1)
Morganella morganii (1)	100	100	*Morganella morganii* (1)
Proteus mirabilis (2)	100	100	*Proteus mirabilis* (2)
Pseudomonas aeruginosa (8)	87.5	100	*Pseudomonas aeruginosa* (7) *Pseudomonas* sp. (1)
Pseudomonas fluorescens (1)	–	100	*Pseudomonas mosselii* (1)
Pseudomonas putida (1)	–	100	*Pseudomonas monteilii* (1)
Salmonella spp. (2)	100	100	*Salmonella* spp. (2)
Serratia marcescens (2)	50	100	*Serratia marcescens* (1) *Serratia* sp. (1)
Staphylococcus aureus (36)	5.5	30.5	*Staphylococcus aureus* (2) *Staphylococcus* sp.(9) *Enterococcus* sp. (2) No reliable ID (23)
Staphylococcus auricularis (3)	–	–	No reliable ID (3)
Staphylococcus capitis (6)	16.7	100	*Staphylococcus capitis* (1) *Staphylococcus* sp.(5)
Staphylococcus epidermidis (93)	26.9	69.9	*Staphylococcus epidermidis* (25) *Staphylococcus hominis* (2) *Staphylococcus* sp.(38) No reliable ID (25) No peaks found (3)
Staphylococcus haemolyticus (11)	36.4	54.5	*Staphylococcus haemolyticus* (4) *Staphylococcus* sp.(2) No reliable ID (5)
Staphylococcus hominis (53)	43.4	79.2	*Staphylococcus hominis* (23) *Staphylococcus lugdunensis* (2) *Staphylococcus* sp.(17) No reliable ID (10) No peaks found (1)
Staphylococcus simulans (3)	33.3	33.3	*Staphylococcus simulans* (1) *Staphylococcus* sp.(1) No reliable ID (1)
Staphylococcus warneri (2)	50	100	*Staphylococcus warneri* (1) *Staphylococcus* sp.(1)
Streptococcus agalactiae (3)	66.7	100	*Streptococcus agalactiae* (2) *Streptococcus gallolyticus* (1)
Streptococcus mutans (2)	–	–	No reliable ID (2)
Streptococcus pneumoniae (1)	100	100	*Streptococcus pneumoniae* (1)
Streptococcus pyogenes (3)	66.7	66.7	*Streptococcus pyogenes* (2) No reliable ID (1)
Streptococcus spp. (3)	–	–	No reliable ID (3)
Total Gram-negative	83.3	96.6	
Total Gram-positive	31.8	64.8	

positive for Gram-negative bacteria was 85% at the species level, and 98.3% at the genus level. In the only enterobacteria in which identification was considered to have failed, there was correlation at the genus species (*Enterobacter amnigenus* in conventional identification vs. *Enterobacter asburiae* in MALDI-TOF), but MALDI-TOF identification was considered to have failed because the score was <1.7.

The only BC positive for *Francisella tularensis* could not be identified by MALDI-TOF MS, because no peptide profiles for this species are included in the BIOTYPER database.

Only three Gram-positive bacilli (one *Listeria monocytogenes*, one *Bacillus fusiformis*, one *Corynebacterium amycolatum*) were studied because their presence in BCs is unusual. There was correlation at the species level for *L. monocytogenes*, at the genus level for *B. fusiformis* (MALDI-TOF from the BC bottle also reported *B. fusiformis*, but the score was <1.9), and identification was considered to have failed for *C. amycolatum*. MALDI-TOF reported *C. amycolatum* too, but the score was <1.7, and thus was considered unreliable.

Enterococci were identified at the species level in 12 of 17 BCs (70.6%), and at the genus level in 13 of 17 BCs (76.5%).

Streptococci were identified in 50% of BCs, both at the species and genus levels. The highest failure rate was for *Streptococcus mutans* and *Streptococcus* spp. (100% failures). Two of three *Streptococcus agalactiae*, two of three *Streptococcus pyogenes* and the only *Streptococcus pneumoniae* found were correctly identified at the species level.

Coagulase negative staphylococci (CNS) showed frequent misidentification at the species level (67.8%), although the correlation at the genus level was higher (123/171; 71.9%). In 48 BCs, CNS grew on plate, but MALDI-TOF identification failed. Twenty-three of them were reported as revealing no reliable identification (14 bottles) or no peaks found (9 bottles). In 25 BCs, identification was considered to have failed because the MALDI-TOF score was <1.7, although the species reported with the highest score was always a CNS.

MALDI-TOF reliability for *Staphylococcus aureus* direct identification was low. Only two of 36 isolates (5.6%) were correctly identified at the species level, and 11 of 36 isolates (30.6%) at the genus level. Two isolates were incorrectly identified as enterococci, and 20 BCs (55.6%) from which *S. aureus* was isolated by conventional methods, were reported by MALDI-TOF MS as revealing no reliable identification.

Current score values accepted (>2 for species identification and >1.7 for genus identification) guarantee excellent identification specificity. Nevertheless, lower values may remain reliable at the genus level. Thirty-one out of 33 (93.9%) Gram-positives with scores in the range 1.5–1.7 (24/25 *Staphylococcus*, 2/2 *Enterococcus*, 4/5 *Streptococcus* and 1/1 *Corynebacterium*) reported the same identification as conventional methods at the genus level. Therefore, we consider those values must be taken into account because they improve Gram-positive genus identification by 12% (Table 2).

Fungaemia was not reliably detected by MALDI-TOF MS. Among 18 BCs positive for *Candida* species, only one (5.6%) was reported correctly at the genus level, whereas, for the other 17 BCs (eight *C. albicans*, eight *C. parapsilosis* and one *C. tropicalis*), MALDI-TOF reported no reliable identification (16 bottles) or no peaks found (one bottle).

When the findings of MALDI-TOF MS and biochemical identification disagreed, 16S rDNA sequencing was performed, confirming the MALDI-TOF MS results in most cases (Table 3).

Discussion

BC processing has improved significantly since the first semi-automated systems were developed in the 1970s, with newer, automated, continuous monitoring systems. Nevertheless, alternative methods for early identification in positive BCs are expensive, and have not reached a wide diffusion so far. In most cases, bacteria growing in BC remain to be identified through subculture on agar plates from BC bottles, directly or after centrifugation, overnight incubation and identification of colonies by rapid or conventional biochemical tests or, in some cases, by fluorescence or *in situ*

TABLE 2. Identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of Gram-positive isolates from blood culture at different identification levels with different scores limits

		Identification level		
	Microorganism	**Species (score >2)**	**Genus (score 1.7–2)**	**Genus (score <1.7)**
Gram-positive	*Streptococci*	6/12 (50%)	6/12 (50%)	10/12 (83.3%)
	Enterococci	12/17 (70.6%)	13/17 (76.5%)	15/17 (88.2%)
	Staphylococci	57/207 (27.5%)	134/207 (64.7%)	158/207 (76.3%)
	Bacillus	0/1 (0%)	1/1 (100%)	1/1 (100%)
	Listeria	1/1 (100%)	1/1 (100%)	1/1 (100%)
	Corynebacterium	0/1 (0%)	0/1 (0%)	1/1 (100%)
	Total	76/239 (31.8%)	155/239 (64.8%)	186/239 (77.8%)

TABLE 3. Comparison between conventional identification, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 16S RNA PCR in identification discrepancies

Conventional identification	MALDI-TOF report (score)	16S RNA PCR identification
Acinetobacter baumanii	*Acinetobacter* spp (score >2)	*Acinetobacter baumanii*
Enterobacter spp.	*Enterobacter hormaechei* (score >2)	*Enterobacter hormaechei*
Klebsiella pneumoniae	*Klebsiella oxytoca* (score >2)	*Klebsiella oxytoca*
Pseudomonas fluorescens	*Pseudomonas mosselii* (score >2)	*Pseudomonas mosselii*
Pseudomonas putida	*Pseudomonas monteilii* (score >2)	*Pseudomonas monteilii/putida*
Staphylococcus aureus	*Enterococcus faecalis* (score 1.7–1.9)	*Staphylococcus aureus*
Staphylococcus aureus	*Enterococcus faecium* (score 1.7–1.9)	*Staphylococcus aureus*
Staphylococcus epidermidis (3 isolates)	*Staphylococcus hominis* (score >2)	*Staphylococcus hominis*

hybridization [6–10]. This methodology delays bacterial identification for several hours, or even days when fastidious bacteria or bacteria requiring complementary tests for identification are involved.

MALDI-TOF MS has been reported as a powerful resource for bacterial identification from conventional culture [11]. Previous studies suggest an excellent correlation between MALDI-TOF MS and conventional microbiological identification in bacterial and fungal clinical isolates [12–16].

Direct analysis of clinical samples might increase the usefulness of MALDI-TOF MS because it might allow clinically useful results earlier than conventional procedures. MALDI-TOF MS direct bacterial identification from positive BC bottles might allow an early species- or genus-oriented empirical treatment. Some studies [17,18] suggest that mortality is 70% lower in patients receiving appropriate empirical treatment.

The use of MALDI-TOF MS directly on clinical samples might have some theoretical limitations (high organisms density required to obtain a good protein profile, interference of human or culture broth proteins in bacterial signature). Nevertheless, studies of MALDI-TOF MS reliability for direct microbial identification in urine specimens study are promising [19]. Recent studies on BCs suggest that this might apply also for BCs [20–22].

The results of the present study show that MALDI-TOF MS allows excellent identification, at the species level, for all the enterobacteria studied, and at the genus level for all the Gram-negatives tested, excepting *F. tularensis* because protein profiles are not available in the BIOTYPER database. Recent studies also show excellent results for Gram-negatives, with correct identification rates >90% [12,16]. When we found any discrepancy, 16S RNA PCR confirmed MALDI-TOF identification in most cases (Table 3). According to previous studies, even 16S rDNA sequencing is not always able to differentiate some *Pseudomonas* species such as *Pseudomonas monteilii* and *Pseudomonas putida* [12]. This happened in the present study for one BC, where *P. putida* was identified by conventional identification methods and *P. monteilii* by MALDI-TOF MS, and 16S rDNA sequencing did not differentiate both species.

It is already described that MALDI-TOF MS identification reliability for Gram-positives is lower than for Gram-negatives in BCs. Stevenson *et al.* [20], among 14 BCs positive for *S. aureus*, reported scores <1.7 in two cases (14.3%) and scores in the range 1.7–1.9 in three cases (21.4%). In nine BCs, *S. aureus* was identified correctly (64.3%). La Scola *et al.* [21] identified correctly *S. aureus* in 40–58% of cases, depending on the protocol used. In the present study only two out of 36 *S. aureus* were identified at the species level (5.5%), and 11 out of 36 (30.6%) at the genus level. Moreover, we obtained two misidentifications at the genus level (*Enterococcus* spp. instead of *Staphylococcus*).

As a whole, the present study identified correctly 55 out of 171 CNS (32.2%) at the species level, and 123 out of 171 (71.9%) at the genus level. Stevenson *et al.* [20] identified, at the species level, 25 out of 42 BCs with CNS (59.5%), and 33 out of 42 (78.6%) with a score of 1.7–1.9. La Scola *et al.* [21] identified 38–93% of CNS depending on the protocol used, and a recent study using ammonium chloride to break erythrocytes membranes identified 26.1% of *S. epidermidis* at the species level, and 82.6% at the genus level [22].

Nevertheless, if we consider correct identifications at the genus level with a score >1.5 instead of >1.7, 158 out of 207 *Staphylococci* BCs were correctly identified at the genus level (76.3%), increasing the genus identifications in 11.6%.

In the case of streptococci, we have identified correctly the 50% by MALDI-TOF MS. The global identification proportion is similar to other studies (40.9%). Nevertheless, if we consider scores >1.5 for genus identification, among the three major pathogens included in the present study (*S. pneumoniae, S. pyogenes* and *S. agalactiae*), five out of six BCs (83.3%) were correctly identified.

We have found no previous studies on MALDI-TOF MS usefulness for detecting fungaemia in BC. The results of the present study show that MALDI-TOF MS, at least with the protocol used, is not a reliable method for detecting fungaemia. In 18 fungaemia episodes, MALDI-TOF MS detected the microorganism in only one BC at the species level. Probable new protocols will be developed in the near future for improving MALDI-TOF reliability in these cases. Indeed, a

recent study [23] reports a new method for detecting invasive fungal infection with good results, although that study was performed with simulated BCs and must be confirmed with real BCs.

In summary, MALDI-TOF MS is a fast and reliable technique for bacterial identification in BCs isolates. Gram-negative bacteria were identified at the genus level with scores >1.7 in 96.6% of cases, and Gram-positives in 64.8%. Moreover, considering correct genus identification with scores >1.5 for Gram-positives, the proportion reaches 77.8%.

Thus, we state that MALDI-TOF MS is an elegible methodology in the clinical setting for straightforward bacterial identification from positive BCs. The identification of invasive fungi directly from BCs is still unsatisfactory, and requires further technical development.

This fast and high accurate technology might allow an early genus-oriented clinical treatment, reducing identification time, costs in laboratory reagents and most likely patient mortality.

Transparency Declaration

Nothing to declare.

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