Direct identification of microorganisms from positive blood cultures using the lysis-filtration technique and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS): a multicentre study

Claudio Farina[1], Fabio Arena[2], Patrizia Casprini[3], Paola Cichero[4], Massimo Clementi[4], Marina Cosentino[1], Roberto Degl'Innocenti[3], Tommaso Giani[2], Francesco Luzzaro[5], Romano Mattei[6], Carola Mauri[5], Maria Nardone[6], Gian Maria Rossolini[2,7,8§], Paula Andrea Serna Ortega[1], Francesca Vailati[1]

[1]Microbiology Laboratories of A.O. "Papa Giovanni XXIII" (formerly "Ospedali Riuniti di Bergamo"), Bergamo;
[2]Department of Medical Biotechnologies, University of Siena, Siena;
[3]Local Health Authority 4, Prato; [4]IRCCS San Raffaele Hospital, Milan;
[5]Manzoni Hospital, Lecco; [6]Campo di Marte Hospital, Lucca; [7]Siena University Hospital, Siena;
[8]Department of Experimental and Clinical Medicine, University of Florence, Florence
[§]Present address: S.O.D. Microbiologia e Virologia, Azienda Ospedaliera-Universitaria "Careggi", Firenze

SUMMARY

Microbial identification from blood cultures is essential to institute optimal antibiotic therapy and improve survival possibilities. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been successfully applied to identify bacteria and yeasts from positive blood cultures broths. The aim of this multicentre study was to evaluate the reliability of the lysis-filtration technique associated with MALDI-TOF MS to directly identify microorganisms from 765 positive blood cultures collected in six Italian hospitals. Overall, 675/765 (78.1%) blood isolates were correctly identified at the species level, with significant differences between Gram-negative and Gram-positive bacteria (92.6%, and 69.8%, respectively). Some difficulties arise in identifying *Streptococcus pneumoniae*, *Staphylococcus aureus*, yeasts and anaerobes. The lysis-filtration protocol is a suitable procedure in terms of performance in identifying microorganisms, but it is quite expensive and technically time-consuming since the time of filtration is not regular for all the samples. The application of the MALDI-TOF MS technique to the direct microbial identification from positive blood cultures is a very promising approach, even if more experience must be gained to minimize errors and costs.

KEY WORDS: Identification, Blood culture, Lysis-filtration technique, MALDI-TOF.

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INTRODUCTION

Sepsis is one of the most important causes of morbidity and mortality all over the world. Rapid microbial identification from blood cultures is essential to promptly institute optimal antibiotic therapy and thus improve survival possibilities (Seifert H., 2009). Therefore, an important task for the microbiology laboratories is to provide expedient reports on positive blood cultures that may guide antibiotic therapy (Søgaard *et al.*, 2007). Decreasing the identification time and anticipating the susceptibility testing results allow adjustments to therapeutic management, and decrease mortality, shorten

Corresponding author
Claudio Farina
USC Microbiologia e Virologia
A.O. "Papa Giovanni XXIII"
Piazza OMS1 - 24127 Bergamo, Italy
E-mail cfarina@hpg23.it

the hospital stay and reduce hospitalization costs (Romero-Gomez *et al.*, 2012).

The first notification of a positive blood culture is typically based on the Gram stain from positive blood culture bottles. Identification is routinely achieved within two days but the presence of fastidious or atypical organisms may lead to longer diagnosis time (Patel *et al.*, 2013).

The key to increase the diagnostic speed is the application of new methods in microorganism identification. In particular, implementation of molecular biology techniques such as PCR, DNA microarrays, sequencing and fluorescent *in situ* hybridization allows a significant increase in diagnostic efficiency and specificity. However, such techniques are particularly expensive and/or require high laboratory qualification (Mancini *et al.*, 2010).

Recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been successfully applied as an identification procedure in clinical microbiology (Bizzini *et al.*, 2010). Thanks to its economic and diagnostic benefits, this innovative technique is now routinely used in laboratory practice (Hrabák *et al.*, 2013). In particular, bacterial identification is obtained within a few minutes on the same day the bacterial colonies grow on the subculture and thus the result is obtained approximately one day earlier than with the conventional procedure (La Scola *et al.*, 2009).

This innovative technique for bacterial identification is usually applied to colonies grown on agar plates but recently some studies refer its use directly on positive blood cultures broths (La Scola *et al.*, 2009; Loonen *et al.*, 2012). However, the presence of blood cells and serum proteins in the samples often hampers the correct identification, since their spectral patterns may overlap. A previous filtration of the samples may lead to cleaner spectra and thus correct, fast diagnosis may be obtained through MALDI-TOF MS.

The aim of the present multicentre study was to evaluate the reliability of the lysis-filtration technique associated with MALDI-TOF MS to directly identify microorganisms from positive blood cultures through a precise comparison with standard procedures.

MATERIALS AND METHODS

Centres

The microbiology laboratories of six Italian hospitals (AO "Papa Giovanni XXIII", formerly "Ospedali Riuniti di Bergamo", Bergamo; Manzoni Hospital, Lecco; Campo di Marte Hospital, Lucca; IRCCS San Raffaele Hospital, Milan; Local Health Authority 4, Prato; Siena University Hospital, Siena) participated in the study. From September 1, 2012 to March 31, 2013, after incubation on a BacT/Alert instrument (bioMérieux sa, Marcy l'Etoile), all consecutively positive BacT/Alert Aerobic FA Plus and Anaerobic FN Plus blood culture bottles collected from hospitalized patients were directly processed. According to an experimental protocol, all samples were subjected to the lysis-filtration technique to directly identify microbes from positive blood cultures by MALDI-TOF MS (VITEK®MS, bioMérieux sa).

Results were compared to those obtained by testing pure colonies obtained after overnight culture, according to standard local procedures.

Analytical protocol

An experimental filtration protocol was used by all the Centres. Selective lysis was implemented to solubilize blood cells, keeping microorganisms intact. Once blood lysis was complete, the microorganisms were collected in a layer on the surface of a filter membrane, and all interfering substances were washed away. The microorganism layer was then scraped off the membrane and deposited directly onto a MALDI-TOF MS target plate for identification. It should be noted that this method is intended for use only with non-charcoal-containing BacT/ALERT® bottles.

The lysis-filtration procedure was as follows:

1. transfer 2.0 mL positive broth to a labelled, screw-capped tube;
2. add the 0.45 μm Millipore Express PLUS 25 mm membrane filters to the 25 mm sintered glass supports of the filter manifold apparatus;
3. add 1.0 mL lysis buffer (0.6% w/v Brij 97, Sigma-Aldrich Co., in 0.4M CAPS Alfa Aesar GmbH & Co, pH 11.7) to the positive broth sample to be tested;
4. cap tube and vortex mix at high speed for 5-7 seconds and start the timer;

5. at minute 2 draw lysed sample into a transfer pipette;
6. begin applying lysate dro wise to the centre of the filter, and continue addition for 40 seconds;
7. allow lysate to be completely pulled into the filter such that no visible liquid remains;
8. apply wash buffer to the respective lysate application areas as they become ready;
9. wash the lysate application area twice more with wash buffer;
10. wash the lysate application area three times with deionized water;
11. harvest the captured microorganisms from the filter surface using a Texwipe CleanTips® swab;
12. hold the swab nearly vertically, tilted slightly toward the direction of scraping, and push it very firmly across the lysate application area in repeated, nearly overlapping stripes;
13. transfer the microorganisms to MALDI-TOF MS target spots, in duplicate;
14. immediately add 1 μL of VITEK MS-CHCA matrix to each spot, pipetting up and down to mix matrix and sample.

If the microorganism is known to be a yeast, mix harvested cells with 1μL of VITEK MS-FA (28.9% formic acid; bioMérieux), and allow to dry before adding VITEK MS-CHCA matrix; 15. proceed with MALDI-TOF MS analysis.

Comparing methods

Each positive blood culture sample was processed as routinely by each laboratory as follows:

1. 5 mL of sample were centrifuged for 5 minutes at 1500 rpm;
2. the supernatant was centrifuged for 10 minutes at 3500 rpm;
3. a drop was used to do a Gram staining;
4. 1 μL of sediment was plated on Columbia agar +5% sheep blood, Columbia CNA agar +5% sheep blood, Chocolate agar + PolyViteX, Mac Conkey agar (MC) and Sabouraud Gentamicin Chloramphenicol 2 agar (SGC2), supplied by bioMérieux;
5. culture media were incubated at 36°C ±1°C overnight;
6. species identification was determined according to standard local procedures.

RESULTS

Overall, 765 consecutively positive BacT/Alert Aerobic FA Plus and Anaerobic FN Plus blood culture bottles (bioMérieux sa) collected from 484 patients were directly processed, including 478 aerobic and 287 anaerobic positive bottles. Table 1 summarizes the global distribution of direct identification results compared with routine techniques. Overall, 675/765 (78.1%) blood isolates were correctly identified at the species level. However, the percentage differed sig-

TABLE 1 - *Global distribution of identification results.*

	No.	*%*
Direct ID = final ID (species level)	538	70.2
Direct ID = final ID (family or genus)	4	0.5
Direct identification missed	138	18.0
Mixed blood culture	48	6.3
Direct ID different from final ID*	14	1.8
Negative blood culture	23	3.2
TOTAL	765	

*In these cases, results obtained directly were discordant with respect to final identification.

TABLE 2 - *Gram-negative bacteria: direct identification vs standard procedures.*

	No.	*Correct ID*	*%*
Gram-negative bacteria			
Escherichia coli	156	151	96.8
Klebsiella pneumoniae	59	58	98.3
Klebsiella oxytoca	4	3	75.0
Proteus mirabilis	1	1	100
Enterobacter cloacae	7	2	28.6
Enterobacter aerogenes	6	5	83,3
Serratia liquefaciens	1	1	100
Serratia marcescens	6	6	100
Stenotrophomonas maltophilia	3	1	33.0
Acinetobacter baumannii complex	1	0	0
Ochrobactrum antropi	1	0	0
Pseudomonas aeruginosa	19	16	84.2
Providencia stuartii	2	2	100
Citrobacter koseri	1	1	100
Hafnia alvei	1	1	100
Campylobacter coli	1	1	100
Morganella morganii	2	2	100
Gram-negative bacteria (total)	271	251	92.6

TABLE 3 - *Gram-positive bacteria: direct identification vs standard procedures.*

	No.	*Correct ID*	*%*
Gram-positive bacteria			
Enterococcus faecalis	34	26	76.5
Enterococcus faecium	12	9	75.0
Streptococcus pneumoniae	14	5	35.7
Streptococcus anginosus	2	1	50.0
Streptococcus bovis	4	1	25.0
Streptococcus gallolyticus	3	2	66.0
Streptococcus mutans	3	0	0
Streptococcus mitis/oralis	1	0	0
Streptococcus intermedius	1	0	0
Streptococcus sanguis	2	0	0
Streptococcus viridans	5	1	20.0
Streptococcus agalactiae	2	2	100
Staphylococcus aureus	90	71	78.9
Staphylococcus epidermidis	132	101	76.5
Staphylococcus auricularis	1	1	100
Staphylococcus capitis	8	4	50.0
Staphylococcus haemolyticus	15	5	33.3
Staphylococcus warneri	4	4	100
Staphylococcus cohnii ssp cohnii	1	1	100
Staphylococcus hominis	44	29	65.9
Micrococcus luteus	1	1	100
Bacillus cereus	2	0	0
Listeria monocytogenes	7	7	100
Gram-positive bacteria (total)	388	271	69.8

nificantly among Gram-negative (92.6%) and Gram-positive bacteria (69.8%).

Table 2 summarizes the results for Gram-negative (enterobacteria and non-fermentative) microorganisms. With few exceptions (e.g., *Enterobacter cloacae* and *Stenotrophomonas maltophilia*), the lysis filtration technique was able to correctly identify these organisms in the large majority of cases.

On the other hand, some difficulties were observed for Gram-positive bacteria. As shown in Table 3, identification of *Streptococcus pneumoniae* and *Staphylococcus aureus* was missed in 64.3% and 21.1% of these strains, respectively.

Table 4 summarizes the discrepancies between the MALDI-TOF MS spot 1 and 2 identifications and the final results obtained from colonies the next day: most of differences were seen in the case of *S. pneumoniae*, alpha-haemolytic streptococci and coagulase-negative staphylococci.

Only eight yeast strains were detected (*Candida parapsilosis*, n=4; *Candida albicans*, n=2; *Candida glabrata* and *Candida krusei*, n=1, each). However, it should be noted that only one *C. parapsilosis* was correctly identified. Identification was missed in the remaining cases.

Similarly, the absolute number of anaerobic bacteria was very limited. Direct identification results were correctly obtained in 50% of cases: 1/2 *Bacteroides thetaiotaomicron*, and 2/5 *Pro-*

TABLE 4 - *Discordant identification.*

Final Identification	*Spot 1 V-MS*	*Spot 2 V-MS*
Candida parapsilosis	*Trichosporon mucoides* *Candida famata*	*Candida lusitaniae*
Enterococcus faecalis	*Streptococcus pneumoniae*	Not performed
Enterococcus faecalis	*Streptococcus pneumoniae*	Not performed
Enterococcus faecalis	*Enterococcus faecium*	*Enterococcus faecalis*
Enterococcus faecalis	*Enterococcus faecium*	*Enterococcus faecalis*
Klebsiella pneumoniae	*Bacillus megaterium*	Not performed
Staphylococcus haemolyticus	*Enterobacter amnigenus* *Gemella sanguinis* *Bacillus licheniformis*	*Gemella sanguinis*
Staphylococcus haemolyticus	*Streptococcus viridans group*	Not performed
Staphylococcus epidermidis	*Staphylococcus hominis*	Not performed
Staphylococcus epidermidis	*Staphylococcus simulans*	Not performed
Staphylococcus epidermidis	*Paenibacillus spp*	*Enterobacter asburiae - Paenibacillus spp* *Haemophilus influenzae - Enterobacter cloacae*
Staphylococcus hominis	*Candida glycolicum*	Not performed
Streptococcus intermedius	*Streptococcus constellatus*	Not performed
Streptococcus mitis/oralis	*Streptococcus pneumoniae*	Not performed

TABLE 5 - *Mixed culture results.*

Mixed cultures	*N°*	*%*
No direct identification	15	31.25
Direct identification of one microorganism	27	56.25
Direct identification of all microorganisms	6	12.5
TOTAL	**48**	

pionibacterium acnes were missed, whereas the one case of *Clostridium histolitycum* was correctly identified.

Considering the results obtained for mixed cultures, it must be noted that only in one third of the cases was identification impossible, but that a partial or a complete identification was gained in 56.2% and 12.5% of cases, respectively (Table 5).

DISCUSSION

The prognosis of sepsis is highly affected by the time when the first blood sample is drawn and positive results are communicated to the clinician. Early and adequate antimicrobial treatment is also essential to reduce mortality (Diekema *et al.*, 2003). Identification by MALDI-TOF MS directly from positive blood culture bottle can significantly change the clinical approach to the treatment of sepsis by starting the optimal therapy or modifying the empirical treatment earlier (Stevenson *et al.*, 2010). In our experience, correct identification at the species level was obtained in 77.5% of cases directly from positive blood culture bottles thus showing a major progress for the therapeutic approach to the management of sepsis.

The MALDI-TOF MS technique proved to be a reliable method, particularly when Gram-negative bacteria are involved as single bacteraemic agents. In this context, direct Gram staining from blood culture positive bottles is essential for guiding bacterial identification (Christner *et al.*, 2010). On the contrary, a careful interpretation must be made if Gram-positive cocci or yeasts are seen at the direct blood sample examination. These limitations agree with previously published data (Seifert H., 2009; Rodriguez-Sánchez *et al.*, 2014).

The lysis-filtration protocol appears a suitable procedure in terms of performance in identifying microorganisms (78.1%) even if the rate of identification is very different between Gram-negative (92.6%) and Gram-positive (69.8%) bacteria. However, it is quite expensive and technically time-consuming, since the filtration time is not regular for all the samples. Inclusion in the workflow of a clinical microbiology laboratory may be difficult depending on local situations and should be compared with other possible technical solutions. Recently, it has been shown that the MALDI-TOF MS technique, using a very-short incubation time of positive blood samples on agar can provide early and reliable bacterial identification without additional time or expenditure. In particular, the mean incubation time till species-level identification was only 2.0 hours for Gram-negative rods and 5.9 hours (shortened to 3.1 hours with a very short ethanol/formic acid protein extraction) for Gram-positive cocci (Idelevich *et al.*, 2014).

This recent experience could increase acceptance among routine laboratories because it is involves no additional cost, fits well into routine procedures and still provides bacterial identification very quickly.

In conclusion, the application of the MALDI-TOF MS technique to the direct microbial identification from positive blood cultures is a very promising approach, even if more experience must be gained to minimize errors and costs.

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