NOVES TECNOLOGIES PER AL DIAGNÒSTIC MOLECULAR DE LA SÈPSIA

Elena Jordana Lluch

2015

Appendix I: Tables

Table 1. Microorganisms included in the molecular assays for the diagnosis of sepsis from positive blood culture

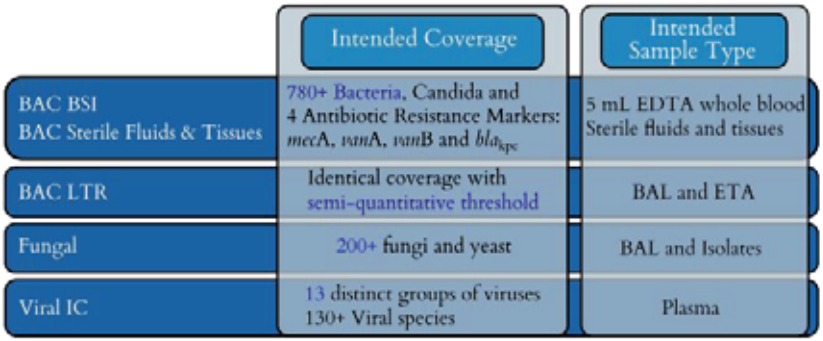
Assay	Gram-positives		Gram-negatives		Fungi	Resistance genes	
Verigene[1]	*Staphylococcus* spp.	*Staphylococcus lugdunensis*	*Acinetobacter* spp.	*Klebsiella oxytoca*		*mecA*	bla_{VIM}
	Streptococcus spp.	*Streptococcus pneumoniae*	*Proteus* spp.	*Pseudomonas aeruginosa*		*vanA*	bla_{IMP}
	Micrococcus spp.	*Streptococcus pyogenes*	*Citrobacter* spp.	*Serratia marcescens*		*vanB*	bla_{OXA}
	Listeria spp.	*Streptococcus agalactiae*	*Enterobacter* spp.			bla_{KPC}	
	Staphylococcus aureus	*Enterococcus faecalis*	*Escherichia coli/ Shigella spp.*			bla_{NDM}	
	Staphylococcus epidermidis	*Enterococcus faecium*	*Klebsiella pneumoniae*			bla_{CTX-M}	
Prove-it Sepsis[2]	*Clostridium perfringens*	*Staphylococcus aureus*	*Acinetobacter baumannii*	*P. vulgaris*	*Candida albicans*	mecA	
	Enterococcus casseliflavus	*S. epidermidis*	*Enterobacter aerogenes*	*Pseudomonas aeruginosa*	*C. dubliniensis*	*vanA*	
	E. faecalis	*Streptococcus agalactiae*	*E. cloacae*	*Salmonella enterica* ssp. *enterica*	*C. glabrata*	*vanB*	
	E. faecium	*S. dysgalactiae* subsp. *Equisimilis*	*Escherichia coli*	*Serratia marcescens*	*C. guilliermondii*		
	E. gallinarum	*S. pneumoniae*	*Haemophilus influenzae*	*Stenotrophomonas maltophilia*	*C. krusei*		
	Listeria monocytogenes	*S. pyogenes*	*Kingella kingae*	*Bacteroides fragilis*	*C. lusitaniae*		
	Propionibacterium acnes		*Klebsiella oxytoca*	*Campylobacter jejuni/ coli*	*C. parapsilosis*		
			K. pneumoniae	*Enterobacteriaceae*	*C. tropicalis*		
			Neisseria meningitidis	*Neisseria* sp. no-meningitidis			
			Proteus mirabilis				
FilmArray	*Staphylococcus* spp.	*S. agalactiae*	*Klebsiella pneumoniae*	*Neisseria meningitidis*	*Candida albicans*	mecA	
	S. aureus	*Enterococcus* spp.	*K. oxytoca*	*Pseudomonas aeruginosa*	*C. tropicalis*	vanA/B	
	Streptococcus spp.		*Serratia* spp.	Enterobacteriaceae	*C. parapsilosis*	bla_{KPC}	
	S. pneumoniae		*Proteus* spp.	*Escherichia coli*	*C. krusei*		
	S. pyogenes		*Haemophylus influenzae*	*Enterobacter cloacae* complex	*C. glabrata*		
			Acinetobacter baumanii				

1. Verigene Blood culture Nucleic Acid Test (Nanosphere, Northbrook, IL, USA), 2. Prove-it Sepsis (Mobidiag, Helsinky, Finlàndia), 3. FilmArray Blood culture Identification (bioMérieux, Marcy l'Etoile, France).

Table 2. Microorganisms included in the different assays for the diagnosis of sepsis from whole blood

Assay	Grampositives		Gramnegatives		Fungi	Resistance markers
SeptiFAST[1]	*Staphylococcus aureus*	*Streptococcus pyogenes*	*Escherichia coli*	*Proteus mirabilis*	*Aspergillus fumigatus*	*mecA* (different assay)
	Staphylococcus epidermidis/ haemolyticus	*Streptococcus agalactiae*	*Klebsiella pneumoniae/oxytoca*	*Pseudomonas aeruginosa*	*Candida albicans*	
	Streptococcus pneumoniae	*Enterococcus faecalis*	*Serratia marcescens*	*Acinetobacter baumannii*	*Candida tropicalis*	
	Streptococcus viridans group	*Enterococcus faecium*	*Enterobacter cloacae/aerogenes*	*Stenotrophomonas maltophilia*	*Candida parapsilosis*	
					Candida krusei	
					Candida glabrata	
Vyoo[2]	*Clostridium perfingens*	*Streptococcus agalactiae*	*Acinetobacter baumannii*	*Morganella morganii*	*Aspergillus fumigatus*	*mecA*
	Enterococcus faecalis	*Streptococcus bovis*	*Bacteroides fragilis*	*Neisseria meningitidis*	*Candida albicans*	*vanA*
	Enterococcus faecium	*Streptococcus dysgalactiae*	*Burkholderia cepacia*	*Proteus mirabilis*	*Candida dubliniensis*	*vanB*
	Staphylococcus aureus	*Streptococcus mutans*	*Enterobacter aerogenes*	*Pseudomonas aeruginosa*	*Candida glabrata*	bla_{CTx-M}*
	Staphylococcus epidermidis	*Streptococcus pneumoniae*	*Enterobacter cloacae*	*Serratia marcescens*	*Candida krusei*	bla_{SHV}*
	Staphylococcus haemolyticus	*Streptococcus pyogenes*	*Escherichia coli*	*Stenotrophomonas maltophilia*	*Candida parapsilosis*	
	Staphylococcus hominis	*Streptococcus sanguinis*	*Haemophilus influenzae* (CTb)	*Prevotella buccae*	*Candida tropicalis*	* Several variants detected
	Staphylococcus saprophyticus		*Klebsiella oxytoca*	*Prevotella intermedia*		
			Klebsiella pneumoniae	*Prevotella melaninogenica*		
Magicplex[3]	*Streptococcus agalactiae*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Klebsiella oxytoca*	*Aspergillus fumigatus*	*mecA*
	Streptococcus pyogenes	*Staphylococcus epidermidis*	*Acinetobacter baumannii*	*Klebsiella pneumoniae*	*Candida albicans*	*vanA*
	Streptococcus pneumoniae	*Staphylococcus haemolyticus*	*Stenotrophomonas maltophilia*	*Proteus mirabilis*	*Candida tropicalis*	*vanB*
	Enterococcus faecalis		*Serratia marcescens*	*Escherichia coli*	*Candida parapsilosis*	
	Enterococcus faecium		*Bacteroides fragilis*	*Enterobacter cloacae*	*Candida krusei*	
	Enterococcus gallinarum		*Salmonella typhi*	*Enterobacter aerogenes*	*Candida glabrata*	

1. SeptiFAST (Roche, Manheim, Germany), 2. Vyoo (SIRS-Lab, Jena, Germany), 3. Magicplex Sepsis Real-Time Test (Seegene, Seoul, South Korea)


Intended Coverage
Intended Sample Type
BAC BSI
BAC Sterile Fluids & Tissues
780+ Bacteria, Candida and 4 Antibiotic Resistance Markers: mecA, vanA, vanB and blakpc
5 mL EDTA whole blood Sterile fluids and tissues
BAC LTR
Identical coverage with semi-quantitative threshold
BAL and ETA
Fungal
200+ fungi and yeast
BAL and Isolates
Viral IC
13 distinct groups of viruses 130+ Viral species
Plasma

Appendix II: Complementary articles

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Establishment of a semi-automated pathogen DNA isolation from whole blood and comparison with commercially available kits

Herbert Wiesinger-Mayr [a,*], Elena Jordana-Lluch [b], Elisa Martró [b,c], Silvia Schoenthaler [a], Christa Noehammer [a]

[a] AIT Austrian Institute of Technology, Molecular Medicine, A-1190 Vienna, Austria
[b] Microbiology Service, "Germans Trias i Pujol" University Hospital, Autonomous University of Barcelona, Badalona, Spain
[c] Biomedical Research Centre Network for Epidemiology and Public Health (CIBERESP), Barcelona, Spain

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ABSTRACT

Molecular methods for bacterial pathogen identification are gaining increased importance in routine clinical diagnostic laboratories. Achieving reliable results using DNA based technologies is strongly dependent on pre-analytical processes including isolation of target cells and their DNA of high quality and purity. In this study a fast and semi-automated method was established for bacterial DNA isolation from whole blood samples and compared to different commercially available kits; Looxster, MolYsis kit, SeptiFast DNA isolation method and standard EasyMAG protocol. The newly established, semi-automated method utilises the EasyMAG device combined with pre-processing steps comprising human cell lysis, centrifugation and bacterial pellet resuspension. Quality of DNA was assessed by a universal PCR targeting the 16S rRNA gene and subsequent microarray hybridisation. The DNA extractions were amplified using two different PCR-mastermixes, to allow comparison of a commercial mastermix with a guaranteed bacterial DNA free PCR mastermix. The modified semi-automated EasyMAG protocol and the Looxster kit gave the most sensitive results. After hybridisation a detection limit of 10^1 to 10^2 bacterial cells per mL whole blood was achieved depending on the isolation method and microbial species lysed. Human DNA present in the isolated DNA suspension did not interfere with PCR and did not lead to non-specific hybridisation events.

1. Introduction

The speed, specificity and sensitivity of pathogen identification are amongst the main advantages of applying molecular detection methods in comparison to conventional cultivation based identification assays (Wellinghausen et al., 2009, Jordan and Durso, 2000). Application of DNA-based technologies to pathogen identification allows accelerated time-to-result thereby fostering earlier initiation of antimicrobial therapy. Several molecular methods have been described for microbe identification, most of which rely on the detection of specific genomic sequences of the infectious pathogen. Amongst these techniques are polymerase chain reaction (PCR), real-time PCR, DNA microarrays and fluorescence in situ hybridisation (FISH). In order to achieve reliable and reproducible results, the applied target DNA must be of high purity and sufficient quantity (Mothershed and Whitney, 2006; Lehner et al., 2005; Shang et al., 2005; Wiesinger-Mayr et al., 2007; Yadav et al., 2005).

A sterile sampling method and an optimised sample preparation protocol are crucial to obtaining PCR compatible DNA targets (Radstrom et al., 2004). Complex biological samples such as blood represent a critical point for specific and sensitive identification of pathogens. Most biological samples contain substances that inhibit subsequent molecular methods (Al-Soud and Radstrom, 2001; Al-Soud et al., 2005). Plasma removal may eliminate interfering proteins, however other cellular blood components such as leukocytes contain high amounts of human DNA and other substances that are suspected to hamper subsequent molecular methods such as PCR. For this reason these cells must be eliminated either by lysis or mechanical removal. However, separation of blood components and blood cells still remains difficult because the success of various strategies is dependent on a number of different factors including plasma viscosity as well as the number and size of cells (Rock et al., 2000; Dzik et al., 2000).

Complete removal of inhibitors is difficult and time consuming. For this reason variations in the reaction mixture of subsequent techniques (e.g. PCR) are applied in an attempt to overcome these effects, e.g. changing enzymes and buffers (Wolffs et al., 2004). Also the addition of PCR enhancers has been shown to be very efficient (Al-Soud and Radstrom, 2000; Wilson, 1997).

In the past major emphasis has been placed on the improvement and acceleration of sample preparation for the application of

* Corresponding author at: AIT Austrian Institute of Technology GmbH, Molecular Medicine, Muthgasse 11, 1190 Vienna, Austria. Tel.: +43 50550 4453; fax: +43 50550 4450.
E-mail address: herbert.wiesinger@ait.ac.at (H. Wiesinger-Mayr).

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molecular diagnostics. Consequently reports have been published recently which demonstrate improved approaches for DNA isolation from faeces, environmental or clinical samples (Nylund et al., 2010; Morin et al., 2010; de Boer et al., 2010). Additionally several ready to use kits have been developed for pathogen DNA extraction from clinical samples. Such systems include the MolYsis kit (Molzym), the Looxster kit (SIRS-Lab), and the DNA isolation protocol of the SeptiFast kit (Roche diagnostics). However, these methods have to be carried out manually and are therefore difficult to implement in routine clinical diagnostic laboratories. On the other hand the EasyMAG is an automated DNA extraction device for human DNA from clinical samples. The recommended initial sample volume is 0.2 mL of whole blood to prevent a possible overload of the DNA binding silica beads with human DNA and other macromolecules. During an EasyMAG run DNA is purified and concentrated by binding to magnetic silica beads. In recent publications the applicability of magnetic silica beads for DNA separation was shown and further optimised. However, these applications still focus on human DNA starting with very small sample volumes (Duarte et al., 2010; Kang et al., 2009).

The human DNA depletion system of the MolYsis kit is based on selective lysis of blood cells using chaotropic buffers with subsequent degradation of released human DNA by chemically resistant DNases. Pathogens remain intact during this working step and are separated from the lysate by centrifugation. Resuspended microbes are then lysed by addition of muralytic enzymes (Disque et al., 2004, 2006). Released pathogen DNA is then purified and enriched using a purification column. The Looxster kit applies a complete cell lysis of whole blood by bead beating. After DNA isolation and purification the human and pathogen DNA are separated from each other. This is enabled by specific binding of pathogen DNA to a matrix contained in an enrichment column. The unbound eukaryotic DNA is eliminated during the subsequent washing steps. Bound bacterial DNA is then eluted and again purified by extraction. The product can then be used for further applications such as PCR. The SeptiFast kit lyses all cells by bead beating of whole blood. Isolated DNA is purified and harvested by application of a purification column. No separation of human and pathogen DNA is carried out.

In general the DNA suspension obtained and analysed in subsequent molecular techniques still contains a background of human DNA and further blood derived macromolecules, which can inhibit PCR. Furthermore the reagents of most suppliers are contaminated with bacterial DNA. This may lead to the generation of false positive results (Mühl et al., 2010; Handschur et al., 2009).

In this study we established and optimised a semi-automated isolation method for pathogen DNA from whole blood. It is based on using an automated EasyMAG device combined with the advantages of the MolYsis kit, which includes a human cell lysis step, human DNA depletion and a fast reduction of sample volume from 5 mL to 0.2 mL. The newly established method was compared to different commercially available kits developed for bacterial DNA isolation from whole blood. All protocols were evaluated with respect to DNA quantity and quality, through the application of sensitivity tests, comprising PCR amplification and subsequent microarray hybridisation. We further compared the amplification efficiency of a guaranteed DNA free PCR mastermix with a well established and sensitive mastermix, which was found to be contaminated with bacterial DNA.

2. Materials and methods

2.1. Strains and preparation of spiked blood

All experiments were carried out using clinical isolates which were obtained by routine culturing of microbes from blood cultures and subsequent commercial microbial identification techniques following the guidelines and procedures of CLSI (Clinical and Laboratory Standards Institute). Pure cultures were obtained by cultivating the respective microbe in Caso bouillon overnight at 37 °C. Bacteria were harvested by centrifugation of 1 mL overnight cultures at 3000 g for 5 min. After discarding the supernatant, the bacterial pellet was washed in phosphate buffered saline (1x PBS) and harvested by centrifugation at 3000 g for 5 min. Microbe concentration per mL was finally adjusted using a McFarland 0.5 standard. Assays were performed using *Escherichia coli* (isolates 68307, 71122), *Staphylococcus aureus* (isolates 12998, 72171), *Klebsiella pneumoniae* (isolate 25809), *Enterococcus faecalis* (isolate 81239) and *Pseudomonas aeruginosa* (isolate 68961).

Fresh whole blood was obtained from the Austrian Red Cross and spiked to final concentrations of 10, 10^2 and 10^3 bacteria per mL. The required volume for each method was then transferred to the prepared vials following the manufacturers' instructions.

2.2. DNA isolation

Bacterial DNA was isolated according to the manufacturers' instructions. The modified EasyMAG isolation included prelysis of blood cells and subsequent transfer of resuspended pellet to the device. Protocols for the different pathogen DNA isolation methods applied from whole blood are described in the following:

2.2.1. Modified EasyMAG DNA isolation

Before sample transfer to NucliSens lysis tubes the sample volume was decreased by lysis of human cells and harvesting bacterial pellet using MolYsis kit for 5 mL whole blood. 5 mL spiked whole blood were pipetted into a sterile 50 ml Falcon tube. 2 mL buffer CM was added, vortexed at full speed for 20 s and incubated at room temperature for 5 min. 2 mL buffer DB1 and 10 µL MolDNase B were added, vortexed for 20 s and incubated at room temperature for 15 min. Bacterial cells were harvested by centrifugation at 11,000 g for 10 min. The supernatant was discarded. The pellet was resuspended in 1 mL buffer RS and transferred to the NucliSens Lysis tubes. Further steps were carried out as described in the EasyMAG bacterial DNA isolation method:

2.2.2. EasyMAG bacterial DNA isolation (BioMerieux, Marcy l'Etoile, France)

NucliSens Lysis tubes for external cell lysis were removed from the refrigerator 15 min before use. 0.2 mL of spiked whole blood was added to the lysis tube and vortexed. Then 140 µL of magnetic silica (BioMerieux) was added to the lysis tube and incubated at room temperature for 10 min. The complete volume was transferred to the vial row and moved into the EasyMAG device. Sample number and volume were registered in EasyMAG software. Parameters such as probe volume (40 µL) and extraction programme (specific B extraction protocol 2.0.1) were set. Ready to use DNA was transferred from vial row to sterile 1.5 mL Eppendorf tubes.

2.2.3. MolYsis Complete (Molzym, Bremen, Germany)

1 mL spiked fresh whole blood was transferred into a sterile 2 mL Eppendorf tube. 250 µL buffer CM was added and vortexed at full speed for 20 s. 250 µL buffer DB1 and 10 µL MolDNase B were added and vortexed immediately for 20 s. The suspension was incubated at room temperature for 15 min. Bacterial cells were harvested by centrifugation at 13,000 g for 10 min. The supernatant was decanted. Cells were washed by adding 1 ml buffer RS to bacteria pellet and vortexing for 10 s followed by centrifugation at 13,000 rpm for 5 min. The supernatant was discarded. 80 µL buffer RL was added and the pellet was resuspended by pipetting. 20 µL BugLysis solution was added and vortexed for 10 s followed by incubation in a thermomixer at 37 °C and 1000 rpm for 30 min. 150 µL buffer RP and 20 µL proteinase K were added and vortexed at full speed for 10 s followed by incubation in a thermomixer at 56 °C and 1000 rpm for 10 min.

250 µL buffer CS was added and vortexed at full speed for 10 s. The DNA was purified by addition of 250 µL binding buffer AB and vortexing at full speed for 10 s. The tube was briefly centrifuged and the lysate was transferred to a spin column. The column was centrifuged at 13,000 rpm for 30 s. The flow through was discarded and 400 µL buffer WB was added to the spin column. The centrifugation was repeated and the flow through was discarded. The column was washed with 400 µL of DNA-free 70% ethanol followed by centrifugation at 13,000 rpm for 3 min. The column was transferred to a 1.5 mL elution tube. 100 µL supplied DNA-free deionised water (preheated to 70 °C) was added to the centre of the column and incubated for 1 min. DNA was eluted by centrifugation at 13,000 rpm for 1 min.

2.2.4. Looxster Plus (SIRS Lab, Jena, Germany)

1 µL solution AF was pipetted onto the wall of a 15 mL centrifugation tube (filled with 5 g beads) and 5 mL spiked fresh whole blood was added. Mechanical lysis was carried out using FastPrep 24 (MP Biomedicals, Illkirch, France) at 6500 m/s rotation speed for 90 s: 45 s rotation–5 min break–45 s rotation. 5 mL buffer A and 100 µL Protease were added, vortexed briefly and incubated at 50 °C for 25 min. The sample was transferred into a 50 mL tube with a purification membrane. Total genomic DNA was isolated by centrifugation at 3000 g for 2 min. The tube was changed, 5 mL buffer B was added onto the membrane and the centrifugation was repeated. 5 mL buffer B was added again and the centrifugation was repeated. The tube was changed, 2.5 mL buffer C was added onto the membrane, incubated at room temperature for 3 min and subsequently centrifuged at 3000 g for 1 min. 2.5 mL buffer C was added again onto the membrane and centrifugation was repeated. The membrane was removed from the 50 mL tube and the flow through was transferred into a 15 mL tube. 4 mL Isopropanol was added. The sample was centrifuged at 5000 g for 30 min at 5 °C. Supernatant was removed and the pellet was washed with 2 mL 70% ethanol (−20 °C). The sample was centrifuged at 5000 g for 5 min at 5 °C. Supernatant was removed and the pellet was dried at 50 °C for 15 min. DNA was resuspended in 300 µL buffer D and dissolved at 50 °C for 30 min. The bacterial DNA was enriched by specific binding of prokaryotic DNA onto the matrix. Therefore 400 µL of the matrix was transferred onto the cartouche and centrifuged at 1000 g for 30 s. The matrix was rinsed with 300 µL buffer D and centrifugation was repeated. The DNA suspension was then transferred onto the matrix. The matrix-DNA mixture was mixed and incubated at room temperature for 30 min. The cartridge was centrifuged at 1000 g for 30 s at RT and the flow through was discarded. 300 µL buffer D was added onto the cartouche, centrifuged at 1000 g for 30 s and the flow through was discarded. The last step was repeated. Bacterial DNA was eluted by addition of 300 µL of buffer E (preheated to 65 °C), mixing and incubation at room temperature for 5 min. The cartouche was centrifuged at 1000 g for 30 s at RT and 300 µL of buffer E was again added onto the cartouche. Centrifugation was repeated. DNA was precipitated by addition of 5 µL solution G, 60 µL 3 M sodium acetate pH 5.2, 480 µL isopropanol and finally vortexing for 10 s. The sample was centrifuged at 16,000 g at 5 °C for 20 min. Supernatant was discarded and the pellet was washed with 1 mL 70% ethanol (−20 °C) and centrifuged at 16,000 g at 5 °C for 5 min. Supernatant was discarded. The washing step was repeated and the pellet was dried at 50 °C. The pellet was dissolved in 30 µL sterile distilled water at 50 °C for 15 min.

2.2.5. SeptiFast DNA isolation protocol (Roche diagnostics, Penzberg, Germany)

The 1.5 mL of whole blood was transferred into 2 SeptiFast Lysis Kit tubes each. Cells were lysed by bead beating in MagNaLyser (Roche diagnostics) at 7000 rpm for 70 s. Lysis suspension was incubated at room temperature for 10 min. Then 150 µL proteinase K was transferred to a 15 mL reaction tube. 1 mL lysis suspension per lysis tube was added to the reaction tube and vortexed. The whole volume of the two vials with lysis buffer was poured into a reaction tube, vortexed and incubated at 56 °C for 15 min. The whole volume of 1 vial Binding Buffer was poured into the reaction tube, vortexed and 3.2 mL of the suspension was transferred to a filter column which was prepared in another reaction tube. The column was centrifuged at 1900 g for 1 min. Then the remaining sample volume was transferred onto the column and centrifuged at 1900 g for 3 min. The reaction tube was changed and one vial Inhibition Removal Buffer was poured onto the filter column. The tube was centrifuged at 4200 g for 2 min. One vial Wash Buffer was poured onto the filter column and centrifuged at 4200 g for 10 min. The tube was changed and centrifugation was repeated at 4200 g for 1 min. The filter column was transferred to another reaction tube and 300 µL of Elution Buffer preheated to 70 °C was pipetted directly onto the centre of the filter column followed by an incubation at room temperature for 5 min. DNA was eluted by centrifugation at 4200 g for 2 min.

2.3. DNA amplification and labelling

The 16S rRNA gene was PCR amplified employing the primer 45fw (5′-GCC TAW CAC ATG CAA GTC GA) and the reverse primer 1391rev (5′-ACG GGC GGT GTG TAC) (Microsynth, Balgach, Switzerland) (0.3 nM in PCR mixture). Primers were designed using the arb software package (Ludwig et al., 2004).

Optimal PCR mixes for a 25 µL reaction volume were found to be ImmoMix (Bioline, Luckenwalde, Germany) and Mastermix 16S Basic kit (Molzym): 12.5 µL of the 2-fold concentrated ImmoMix was mixed with 0.5 µL of each primer. Different DNA sample volumes (1, 2, 5 and 10 µL) were tested and optimal volume was found to be 5 µL, which was added and the mixture was diluted with 5.5 µL PCR water (Bioline) to obtain the final 25 µL reaction volume. To obtain a PCR mixture based on the Mastermix 16S Basic kit, 10 µL of the 2.5-fold concentrated mixture was mixed with 0.5 µL of each primer, 0.8 µL polymerase, the appropriate volume of DNA suspension (5 µL) and finally adjusted with PCR water to a final volume of 25 µL.

PCR cycling included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min using a Biometra T3000 Thermocycler (Goettingen, Germany). Temperature cycles were terminated at 72 °C for 10 min to complete partial amplicons, followed by storage at 4 °C until further usage.

Successful amplification was confirmed by resolving the PCR products on a 1.5% agarose gel (SeaKem, Biozym, Vienna, Austria) with ethidium bromide in TBE buffer (0.1 M Tris, 90 mM boric acid, 1 mM EDTA) (Invitrogen, Paisley, UK).

In order to check the lowest detection limit without any identification of the pathogen a primer pair was used to amplify a fragment of the 16S rRNA gene. An amplicon of about 300 bp was generated employing the primer 45fw and the reverse primer 347rev (5′-TTC CCY ACT GCT GCC TC) (Microsynth, Balgach, Switzerland) (0.3 nM in PCR mixture). The additional primer was also designed using the arb software package. Reaction conditions applied were the same as described above.

PCR products were labelled using a primer extension method: 6 µL of PCR product was used for labelling in the primer extension reaction mix, which contained 0.9 mM each of forward primer 45fw (16 S rRNA), 1.5 U Vent (exo) polymerase (New England Biolabs, Ipswich, UK), 3 mM $MgSO_4$ and 50 µM of dATP, dGTP, and dTTP, 25 µM of dCTP and 25 µM Cy3-dCTP (Amersham Biosciences, UK). The reaction mix was cycled 25× at 95 °C, 60 °C and 72 °C each at 20 s followed by a final extension step for 5 min at 72 °C. Temperature cycles were preceded by a 3 min incubation at 95 °C.

2.4. Microarray preparation, hybridisation and read-out

Microarray probes were designed using the arb software package. Microarray layout and fabrication was realised as previously described (Wiesinger-Mayr et al., 2007).

Prior to hybridisation of the microarray, slides were pre-treated with blocking buffer (3 M urea, 0.1% SDS) at room temperature for 30 min in order to inactivate reactive groups on the slide surface. Slides were rinsed in 1x PBST (1x PBS containing 0.1% Tween) for 5 min and in distilled water prior to drying under airstream.

The hybridisation mixture had a final volume of 30.3 µL. It contained 24 µL of the labelled DNA reaction mixture and was adjusted using 20x SSC and 10% SDS to a final concentration of 4x SSC and 0.1% SDS. The suspension was denaturated at 95 °C for 5 min and finally a hybridisation control (BSrev: 5′ end Cy3-labelled oligonucleotide sequence: AAG CTC ACT GGC CGT CGT TTT AAA) was added at a final concentration of 0.15 nM. The hybridisation control also served as a guiding dot on the microarray for the later microarray read-out and had been shown not to interfere with other detection probes. A total volume of 22 µL of the hybridisation mix was transferred to a cover slip (22 × 22 mm) and subsequently applied to the microarray surface. Hybridisation was realised at 65 °C in a vapour saturated chamber for 1 h. Slides were washed in 2x SSC and 0.1% SDS for 5 min followed by 0.2x SSC for 2 min and 0.1x SSC for 1 min. Slides were dried by centrifugation at 900 g for 2 min.

Slides were scanned at a resolution of 10 µm with an Axon Genepix 4000A microarray scanner (Axon, Union City, California) at equal laser power and sensitivity level of the photomultiplier (650 pmt) for each slide. Obtained images were analysed using the Genepix software and the resulting gpr-files were used for further analysis.

2.5. Assessment of clinical specimens

A total of eight patients with a clinical suspicion of sepsis were included. Blood samples for conventional pathogen detection were withdrawn directly in blood culture bottles (BacT/ALERT FA and BacT/ALERT FN, BioMérieux, Marcy l'Etoile, France) and transferred to the blood culture automated system (BacT/ALERT 3D Microbial Detection System, BioMérieux). Etiologic agents were identified from positive blood cultures following standard microbiologic procedures, including Gram staining and Vitek 2 Compact identification and antibiogram (BioMérieux).

For molecular detection of pathogen whole blood specimens, that had been collected in EDTA tubes (Becton–Dickinson, Franklin Lakes, NJ, USA) around the time of blood culture inoculation for the purpose of routine analyses, were used and treated anonymously. A 5 mL aliquot of each tube was transferred into a 10 mL sterile tube and the DNA isolation procedure was carried out as described in Section 2.2. "Modified EasyMag protocol" followed by DNA detection using microarray technology as described in Sections 2.3 and 2.4.

3. Results

3.1. Comparison of DNA concentration and purity

DNA concentration and purity was determined using the NanoDrop ND-1000. The DNA suspension obtained using the modified EasyMAG method had an average ratio (260/280 nm) of 1.32, indicating that high concentrations of other macromolecules such as proteins were still present in the sample (Fig. 1A). The comparison with other kits showed that the highest purity of DNA was obtained applying the Looxster kit and the Roche isolation method included in SeptiFast. Here the averages of the measured ratios were 1.86 and 1.73, respectively. The isolation method MolYsis had an average DNA purity of 1.43. The majority of the DNA measured in the samples was human DNA since bacteria were spiked to the blood only at a low concentration and were likely undetectable. The modified EasyMAG protocol yielded, on average, a DNA concentration of 16.6 ng/µL. The low total amount of DNA suggests that the pre-lysis step for human cells had a strong impact on decreasing the DNA concentration in the final eluate. The high amount of total DNA of 99.6 ng/µL of the

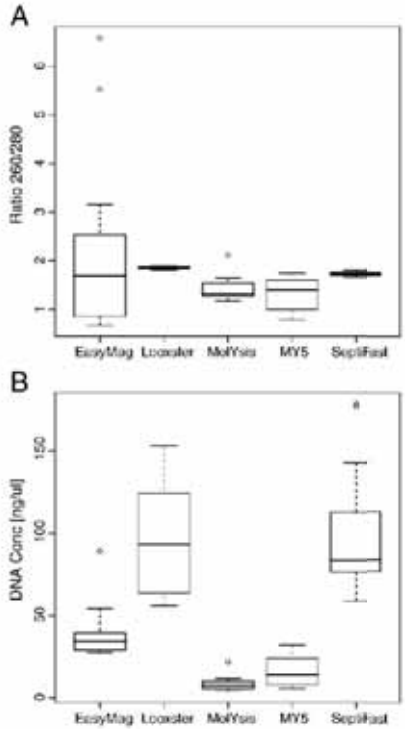

Fig. 1. (A) Purity of DNA suspension was determined by measuring adsorption ratio of 260 to 280 nm. The boxplot shows that the highest purity with low variations was obtained using the Looxster and SeptiFast kit. Applying standard EasyMAG protocol two outliers was observed with a ratio of 6.6 and 5.5. The initially applied blood volume per method was: modified EasyMAG protocol (MY5): 5 mL, Looxster: 5 mL, MolYsis: 1 mL, SeptiFast isolation method: 3 mL, and EasyMAG: 0.2 mL. (B) Boxplot of total DNA concentration (ng/µl) in obtained suspension. The MolYsis kit and the modified EasyMAG protocol (MY5) had the lowest DNA concentration.

SeptiFast method and 97.4 ng/µL of the Looxster kit suggests a high concentration of human DNA in the sample (Fig. 1B). The lowest DNA concentration of 8.71 ng/µL was obtained by the MolYsis kit indicating good separation of human DNA. The standard EasyMAG protocol using 0.2 mL whole blood yielded an average DNA concentration of 41.1 ng/µL, indicating a high concentration of background DNA considering the small amount of sample applied.

The yield of total DNA obtained per mL of blood indicates a clear effect of the human DNA depletion (Table 1). The SeptiFast isolation protocol and the standard EasyMag protocol yielded the highest amounts of total DNA (9960 and 8220 ng per mL whole blood respectively). Conversely, the protocols utilising depletion of human DNA led to lower DNA yields per mL blood. Samples processed with the MolYsis kit, showed a low DNA concentration in the final suspension volume. However, the DNA yield is greatly increased when considering the applied blood and elution volume, since MolYsis utilised a small sample volume (1 mL whole blood) and was eluted in a greater volume (100 µL), resulting in a total DNA yield of 871 ng per mL blood. The Looxster kit gave a total DNA yield of 584 ng per mL blood. The lowest DNA concentration (132.8 ng per mL blood) was obtained with the modified EasyMag protocol. The difference in DNA concentration and absolute DNA yield compared

Table 1
Comparison of total DNA amounts obtained by each isolation method and DNA amounts obtained from 1 mL blood.

	Blood vol. [mL]	Elution vol. [µL]	Average DNA conc. [ng/µL]	Total DNA amount [ng]	Tot. DNA per mL blood [ng]	Hum. DNA depletion
EasyMag	0.2	40	41.1	1644	8220	No
Modified EM	5	40	16.6	664	132.8	Yes
MolYsis	1	100	8.71	871	871	Yes
Looxster	5	30	97.4	2922	584.4	Yes
SeptiFast mol.	3	300	99.6	29,880	9960	No

to other isolation methods was a consequence of the high initial blood volume and the low volume of elution buffer.

3.2. Amplification of bacterial DNA

Two different mastermixes for DNA amplification were used: ImmoMix and 16S Basic kit. Several tests of these different mastermixes using DNA from pure bacterial cultures demonstrated that the ImmoMix yielded the greatest amplification efficiency, as evaluated by agarose gel electrophoresis (data not shown). However, when using PCR grade water in the negative control and primers amplifying short fragments in order to maximise the sensitivity of detection, non-specific amplification was observed in all negative controls. For this reason, the 16S Basic kit was chosen for the testing of blood samples as it was the only PCR kit guaranteed to be free of bacterial DNA. The direct comparison of 16S Basic kit with ImmoMix using the DNA samples isolated from spiked whole blood showed that the 16S Basic kit was as sensitive as ImmoMix due to an optimisation of 16S Basic mastermix for use with a high background of interfering human macromolecules. Furthermore amplification evaluation by gel electrophoresis indicated that the Basic kit PCR produced discrete bands whilst the ImmoMix kit often led to the generation of ambiguous bands and smears (Fig. 2). Hybridisation of these PCR products on the microarray also resulted in more non-specific signals than were obtained when using the other PCR mix.

Amplification, evaluated by gel electrophoresis, revealed different detection limits dependent on the isolation method applied and the bacterial species spiked into whole blood. It was shown that the modified EasyMAG protocol was generally the most sensitive with detection limits as low as 50 bacteria per mL blood (e.g. *E. coli*) followed by the sensitivity of the Looxster kit. When investigating *P. aeruginosa* DNA, a clear band was detected using agarose gel electrophoresis at 10^2 bacteria per mL blood using the Looxster kit, SeptiFast isolation and modified EasyMAG. Based on MolYsis and EasyMAG a detection limit after PCR of 10^3 cells per mL blood was indicated (Table 2). The best results were obtained from DNA isolations with *E. coli* cells.

3.3. Microarray hybridisation

The microarray used comprised 75 different species specific probes, two positive controls (PCR and hybridisation-control) and one negative control (buffer spot). Independent of the applied DNA isolation method a high background of human DNA did not interfere with hybridisation resulting in species specific signal patterns (data not shown).

Detecting closely related species the signals obtained by cross-hybridisation onto multi-specific probes were detected to the same extent as during hybridisation with DNA from pure cultures. During all experiments no false positive results were generated, showing no influence of the high human DNA background. False negative results were only obtained from hybridisations below the detection limit of the corresponding method. Bloodstream infections caused by more than one species may be detected. Thus a combination of Gram positive (*S. aureus*) and Gram negative (*K. pneumoniae*) species were tested. The parallel detection of two pathogens in one sample generated a combination of the individual hybridisation patterns, which were obtained by single species identification (Fig 3).

The semi-automated EasyMAG protocol resulted in the most sensitive detection of Gram negative species. Evaluating signal patterns only low signal intensities from probes of very closely related species were obtained (Table 3). However, signals from specific probes were on average 48% lower than that obtained from pure cultures. Considering both Gram negative and Gram positive species, maximal signal intensities were obtained after hybridisation of samples processed with the Looxster kit particularly for Gram-positive organisms. Concentrations of 10^2 cells per mL blood resulted in strong signal intensities with all methods and species. Concentrations of 10^1 bacteria per mL blood were only detected using the MolYsis (for *E. faecium* and *S. aureus* [weak signals]), Looxster kit (for *E. faecium* and *S. aureus*), modified EasyMAG protocol (for *E. coli*, *P. aeruginosa* and *S. aureus* [weak signals]) and SeptiFast isolation protocol (for *E. faecium*, *S. aureus* and *E. coli* [weak signals]). The lowest sensitivity was obtained through application of standard EasyMAG protocol with the recommended starting volume of 0.2 mL blood. Whereby a detection limit of 10^3 was found in best case for *E. faecium* and *P. aeruginosa*.

3.4. Analysis of clinical samples

Eight different patient blood samples were analysed in a clinical diagnostic laboratory. A water negative control was included in each

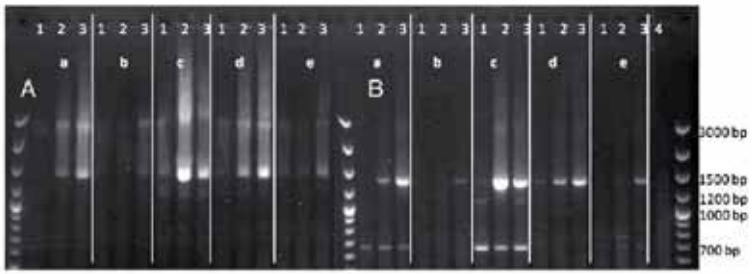

Fig. 2. Gel electrophoresis image of PCR amplicons derived from *P. aeruginosa* DNA isolated from whole blood. Two PCR mastermixes were compared: ImmoMix (A) and 16S Basic kit (B). A bacterial dilution row was spiked into whole blood: lane 1: 5×10^1 bacteria/mL blood, lane 2: 10^2 bacteria/mL blood and lane 3: 10^3 bacteria/mL blood. Bacterial DNA was isolated applying five different techniques: SeptiFast isolation kit (a), MolYsis complete (b), Looxster (c), modified EasyMAG protocol (d) and EasyMAG protocol (e). Lane 4: negative control.

Table 2
Detection limits displayed as bacterial cells per mL whole blood after evaluation of PCR efficiency by gel electrophoresis.

	SeptiFast	MolYsis	Looxster	MYS [a]	EasyMAG
P. aeruginosa [b]	10^2	10^3	10^2	10^2	10^3
E. coli [b]	5×10^1	10^2	10^3	10^2	10^1
S. aureus [b]	10^3	10^2	5×10^1	5×10^1	10^3
E. faecium [b]	10^2	10^2	5×10^1	5×10^1	10^2
K. pn. + S. aur [b, c]	5×10^1	5×10^1	10^1	10^1	10^3

[a] It was shown that in average lowest detection limits were obtained by modified EasyMAG protocol.
[b] The dilution row was realised in order to obtain the following concentrations: 10^3, 10^2, 5×10^1, and 10^1 bacterial cells per ml. blood. 5 µl. of DNA suspension was transferred into each PCR mix.
[c] Abbreviations: K.pn.–*K. pneumoniae*, S. aur.–*S. aureus*.

extraction run. In parallel to the molecular detection and identification using our microarray, a blood culture was also carried out and conventional microbiological methods were used for identification. Those results were directly compared to those obtained by DNA isolation using modified EasyMag protocol combined with microarray based species identification. The microarray used had been previously designed, in-house, to identify 25 different pathogen species including both Gram positives and Gram negatives (Wiesinger-Mayr et al., 2007).

Using blood culture certain pathogens could be detected in five of the patient samples (Table 4). In three samples the pathogens could be identified correctly by both conventional microbiological methods and the molecular assay as *E. coli*. The species *Listeria monocytogenes* and *Streptococcus salivarius* were not detected using the molecular assay, since no species-specific probes for these bacteria were included in the microarray. Three patient samples were found negative for pathogen by both methods.

3.5. Simplicity of isolation protocol

The modified EasyMAG protocol was carried out within 75 min requiring hands-on-times of approximately 10 to 15 min to process five to eight specimens. The device was adjusted to elute 40 µl. of DNA suspension which could be directly used for subsequent molecular assays. Using microarray detection 10 bacteria per mL blood could be

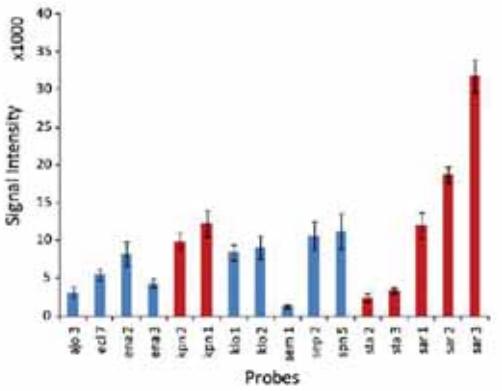

Fig. 3. Signal intensities and hybridisation pattern after detection of two pathogens in one blood sample. One hundred cells of *S. aureus* and *K. pneumoniae* each were added to the sample. Red bars are specific and blue bars are unspecific signals, obtained from probes for closely related species (e.g. *K. oxytoca* and *E. aerogenes*). Applied isolation method: modified EasyMAG protocol. Abbreviations of probe names: ajo: *A. johnsonii*, ecl: *E. cloacae*, ena: *E. aerogenes*, kpn: *K. pneumoniae*, klo: *K. oxytoca*, sem: *S. marcescens*, sep: *S. epidermidis*, spn: *S. pneumoniae*, sta: *Staphylococcus*, and sar: *S. aureus*.

Table 3
Average signal intensities after microarray hybridisation of *E. coli* DNA.

	eco2	eco3	clk2	ecl7	pmi3	sem2	ajo3	alw1	spn5
Pure culture	24	20	5	2	6	2	3	2	1
Mod. EasyMAG	16	11	1	0	1	0	0	0	0
MolYsis	14	12	0	0	1	0	0	2	0
Looxster	10	7	0	0	1	0	0	1	0
SeptiFast	5	4	0	0	1	0	0	2	0
EasyMAG	1	0	0	0	0	0	0	0	0

No additional signals were obtained by the high background of human DNA. Signal patterns of samples isolated from blood were similar to those isolated from pure cultures. Different concentrations of bacterial cells were spiked into whole blood. Displayed values are the mean values of absolute signal intensities of different hybridisations. Hybridisation of labelled DNA from pure cultures resulted in stronger signal intensities but also in an increase of signals from probes for closely related species. Displayed signal values were divided by 1000 for better visualisation.
Abbreviations of probe names: eco: *E. coli*, clk: *C. koseri*, ecl: *E. cloacae*, pmi: *P. mirabilis*, sem: *S. marcescens*, ajo: *A. johnsonii*, alw: *A. hoffii*, spn: *S. pneumoniae*.

detected (*E. coli*). Since 5 mL of blood was used, the maximal amount of bacterial genomic DNA copies in the eluate corresponded to 50 bacteria.

4. Discussion

For all molecular pathogen identification methods an efficient isolation from the clinical sample that results in microbial DNA template of high purity is important to achieve accurate results. The removal of inhibitory components from the sample is a major focus of target isolation strategies. Sensitivity of sequence specific amplification and detection methods requires an efficient DNA isolation protocol. Furthermore methods for clinical application need to be rapid, and simple, allowing the potential for automation.

Therefore a semi-automated method based on a device which is already widely available in clinical laboratories was established and its efficiency was compared with different commercially available methods for whole blood bacterial DNA isolation. The new method is rapid and significantly reduces both hands-on-time and contamination risk.

Each method examined utilised a different initial volume of whole blood. It was shown that the methods Looxster and SeptiFast with a high initial volume of 5 and 3 mL whole blood, respectively, gave the highest background DNA concentration. However, the effect of human DNA depletion with the Looxster kit could be clearly shown in comparison with the SeptiFast based isolation method. The method MolYsis and standard EasyMAG protocol utilised a lower initial blood volume of 1 and 0.2 mL, respectively. As such the remaining human DNA in the final suspension was considerably lower. However, the established semi-automated method based on modified EasyMAG protocol combined with MolYsis' human cell pre-lysis utilised a higher initial blood volume of 5 mL and still resulted in a low

Table 4
Comparison of pathogen identification results using blood cultures and the molecular assay based on modified EasyMag protocol. Abbreviation: BC–blood culture.

Sample	BC results	Molecular assay
1	*Listeria monocytogenes*	Negative
2	*Escherichia coli*	*Escherichia coli*
3	*Streptococcus salivarius*	Negative
4	Water control	Negative
5	*Escherichia coli*	*Escherichia coli*
6	*Escherichia coli*	*Escherichia coli*
7	Negative	Negative
8	Negative	Negative
9	Negative	Negative
10	Water control	Negative

concentration of human DNA. The resulting concentration was comparable to those of MolYsis and standard EasyMAG. Due to the high starting blood volume and the low volume of elution buffer (40 µL) the modified EasyMag protocol resulted in the lowest DNA amount obtained per mL blood. Interestingly the same reagents as for the MolYsis kit were applied with the difference of using larger tubes (50 mL Falcons) and higher reagent volumes. A possible overload of the silica beads, which would lead to a loss of DNA, can be excluded since the standard protocol yielded much higher DNA amounts than the modified protocol. In terms of purity of the final suspension (ratio 260/280) the Looxster and SeptiFast methods were the most reproducible. In comparison to those kits the modified EasyMAG protocol demonstrated a lower DNA purity. Comparison of the commercially available kits partially confirmed the results of a previous study; Hansen et al. (2009) showed highest sensitivity for the MolYsis kit. However other methods were not carried out corresponding to the manufacturers' instructions (e.g. Looxster), the influence of sample volume (of spiked whole blood) on detection limit of fungal DNA has also been previously described, whereby an increase of blood volume from 1 to 3 mL resulted in an increase of sensitivity in three of four general methods for DNA isolation (Schulz et al., 2009).

The optimal volume of DNA suspension added to the PCR mix was determined by comparing different volumes added: 1, 2, 5 and 10 µL. It was shown that for all methods the best results were obtained using 2 or 5 µL of DNA suspension in a 25 µL PCR reaction volume, thus 5 µL DNA suspension was used for subsequent experiments. In order to allow differentiation of all targeted bacteria the almost complete 16S rDNA sequence was amplified (position 45 to 1391). However, amplifying a large fragment during PCR results in decreased sensitivity compared to generation of short fragments. The amplification of a shorter fragment of 16S rDNA (approx. 300 bp) led to a 10 times lower detection limit for some tested methods and species. However, using short fragments prevents the discrimination of some isolates down to species level. Applying a highly efficient PCR mix (Immomix, Bioline) led to failure of negative controls. Therefore this kit was compared with the 16S basic kit (Molzym) which is guaranteed free of bacterial DNA. Using DNA from pure cultures the Immomix led to stronger bands on the agarose gel, however PCR amplicons were detected in almost all negative controls and indicating unreliable results. Applying DNA isolated from whole blood samples the 16S basic kit gave similar amplification efficiencies as the Immomix. Nevertheless negative controls using the 16S basic kit were consistently clean. When considering the microarray signal intensities, amplicons generated by the 16S basic kit led to stronger and more specific signals than when the Immomix kit was used.

The results of this study were mainly obtained using spiked blood samples. This fact might lead to a systematic error of the results, since real clinical samples not only contain viable cells but also dead bacteria and free bacterial DNA. This free bacterial DNA will not be considered by certain methods. The Looxster and SeptiFast isolation methods lyse all cells present in whole blood during the first working step thus also the free DNA. The MolYsis method employs an initial partial lysis step for eliminating human cells and its DNA. During this working step again the free bacterial DNA is removed. Since this step is also applied for the modified EasyMAG protocol the free bacterial DNA would not be available for subsequent diagnostic methods. Depending on the concentration of such free DNA in patient's samples this fact might represent a disadvantage of these methods. However recent publications of clinical studies of the MolYsis kit reveal a good sensitivity of the manual method applied on clinical samples (Wellinghausen et al., 2009). Although a small number of clinical specimens were also tested in the present study, a clinical validation study including a wider variety of sepsis-related pathogens is currently being planned in order to assess the usefulness of the modified EasyMAG protocol in the clinical setting.

Amongst the most important aspects of molecular detection methods in clinical routine are protocol duration, hands-on-time, ease of use and risk of contamination. Regarding these criteria the semi-automated method is well suited to clinical application since the total isolation can be completed within 70 min and requires minimal manual working steps including the pre-processing steps for human cell lysis, DNA degradation, pellet resuspension and transfer into the EasyMAG device. Thus the risk of contamination using this method was also the lowest compared to the other protocols. The second fastest method was the isolation performed during SeptiFast analysis, which took about 80 min, however this includes several manual working steps and centrifugation. An advantage of this method was the decrease of contamination risk due to the avoidance of pipetting steps. The MolYsis protocol could be performed within 3 h including a DNA purification step using a column. The method, which required the most hands-on-time combined with longest time-to-result, was the Looxster method, which required 7 h until the DNA suspension was obtained. Considering the requirements of clinical laboratories, these manual methods are not well suited for routine diagnosis since they are time consuming and comprise labour-intensive manual working steps which require a full-time and skilled laboratory staff. Along with the manual working steps the contamination risk increases correspondingly.

Automation of the method is well suited to clinical laboratories. The described semi-automated EasyMAG method is a significant step in this direction, allowing sensitive detection and identification of pathogens directly from clinical samples. For a recent publication a research group used microfluidic approaches for semi-automated bacterial DNA isolation from whole blood. However, also for this method a pre-processing manual lysis step was carried out prior to application to the device and the applied sample volume was small (100 µL) (Mahalanabis et al., 2009).

5. Conclusion

In this study we have established and optimised a semi-automated method for bacterial DNA isolation from whole blood and compared it to currently available methods (all manual protocols with several hours of working time).

The newly established method is based on the EasyMAG device which is already routinely used in many clinical laboratories. Thus we propose the adaption of this existing tool to the needs of molecular infection diagnostics as it circumvents the shortcomings of currently available protocols and will contribute to an improvement in patient treatment.

Furthermore other automated DNA isolation devices are available or are already in use in clinical routine, which are even less cost-intensive. Therefore other automated methods may be tested for fast isolation of bacterial DNA directly from clinical samples. Application of an appropriate pre-lysis step for human cell depletion which also includes sample volume reduction has the potential to increase sensitivity even when using already established isolation tools by simple process adaptation.

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Revisión

La espectrometría de masas en el laboratorio de microbiología clínica

Elena Jordana-Lluch[a], Elisa Martró Català[a,b] y Vicente Ausina Ruiz[a,c,*]

[a] Servicio de Microbiología, Fundación Instituto de Investigación en Ciencias de la Salud Germans Trias y Pujol, Hospital Universitario Germans Trias y Pujol, Universidad Autónoma de Barcelona, Badalona, Barcelona, España
[b] CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona, España
[c] CIBER Enfermedades Respiratorias (CIBERES), Bunyola, Mallorca, España

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Identificación microbiológica

RESUMEN

Actualmente, las enfermedades infecciosas siguen causando una elevada mortalidad y morbilidad. Los métodos de diagnóstico microbiológico se basan en el cultivo seguido de la identificación fenotípica del microorganismo una vez aislado, y el tiempo necesario para su obtención puede variar de 24 a 48 h. Dado que la identificación microbiológica repercute directamente en el manejo del paciente y su pronóstico, son necesarias nuevas herramientas diagnósticas capaces de detectar e identificar cualquier microorganismo de manera rápida y fiable. A lo largo de los últimos años se han desarrollado diferentes técnicas moleculares basadas en la amplificación genética con el objetivo de reducir el tiempo necesario para la identificación de los microorganismos implicados en diferentes tipos de procesos infecciosos. Por otro lado, la espectrometría de masas ha surgido como una alternativa rápida y eficaz a los métodos convencionales para la identificación de microorganismos. En esta revisión se describe la tecnología en sus dos formas más utilizadas —desorción/ionización por láser asistida por matriz (MALDI-TOF) e ionización por electrospray (ESI-TOF)— para el análisis tanto de las proteínas como de los ácidos nucleicos microbianos, así como las diferentes plataformas comerciales disponibles. Así mismo, se hace una revisión de los trabajos de mayor interés en microbiología clínica.

Mass spectrometry in the clinical microbiology laboratory

Keywords:
Mass spectrometry
Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)
PCR-MALDI-TOF
PCR-electrospray ionization time-of-flight (PCR-ESI-TOF)
Identification of microorganisms

ABSTRACT

Infectious diseases are still a cause of high mortality and morbidity rates. Current microbiological diagnostic methods are based on culture and phenotypic identification of isolated microorganisms, which can be obtained in about 24–48 h. Given that the microbiological identification is of major importance for patient management, new diagnostic methods are needed in order to detect and identify microorganisms in a timely and accurate manner. Over the last few years, several molecular techniques based on the amplification of microbial nucleic acids have been developed with the aim of reducing the time needed for the identification of the microorganisms involved in different infectious processes. On the other hand, mass spectrometry has emerged as a rapid and consistent alternative to conventional methods for microorganism identification. This review describes the most widely used mass spectrometry technologies —matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and electrospray ionization time-of-flight (ESI-TOF)—, both for protein and nucleic acid analysis, as well as the commercial platforms available. Related publications of most interest in clinical microbiology are also reviewed.

Introducción

A pesar de los avances en el campo de la medicina, las enfermedades infecciosas siguen siendo la causa de una elevada mortalidad y morbilidad. Según el informe actualizado en 2011 de la Organización Mundial de la Salud, 4 de las 10 principales causas de muerte a nivel mundial están causadas por agentes infecciosos, siendo estas las infecciones de las vías respiratorias bajas (6,1%), la diarrea (4,3%), la infección por el VIH (3,1%) y la tuberculosis (2,4%). En países subdesarrollados, a estas se les suman la malaria (5,2%) y las infecciones neonatales (2,6%)[1].

Para un óptimo manejo de los pacientes es indispensable obtener la identificación microbiológica del agente causal de la infección en el menor espacio de tiempo posible. Solo así será posible aplicar

* Autor para correspondencia.
Correo electrónico: vausina.germanstrias@gencat.cat (V. Ausina Ruiz).

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un tratamiento antibiótico efectivo o reducir el espectro del tratamiento administrado empíricamente, disminuyendo la aparición de resistencias[2].

Sin embargo, la obtención de un diagnóstico microbiológico rápido resulta compleja utilizando los métodos convencionales considerados como *gold standard*, que se basan en el cultivo seguido de la identificación fenotípica del microorganismo una vez aislado[3]. Aunque la obtención de un cultivo puro del microorganismo implicado en una infección permite no solo la identificación del mismo, sino también el estudio de su sensibilidad a diferentes antibióticos y la determinación de marcadores epidemiológicos, el tiempo necesario para su obtención puede variar entre 24 y 48 h, según los requerimientos del microorganismo. Así mismo, serán necesarias 24 h adicionales para disponer de los resultados definitivos de la identificación y el antibiograma, lo que supone como mínimo un total de 48 a 72 h desde la obtención de la muestra del paciente.

Este periodo de tiempo puede reducirse utilizando instrumentos que ofrecen una identificación automatizada basada en el perfil bioquímico de los microorganismos. Uno de los sistemas más utilizados de identificación automatizada es el Vitek 2 Compact (bioMérieux, Marcy l'Etoile, Francia), que permite tanto la identificación de bacterias y levaduras como la obtención de su patrón de susceptibilidad a diversos antibióticos y antifúngicos en unas 6-12 h partiendo del cultivo puro[4]. Otros sistemas también utilizados para esta finalidad son MicroScan (MicroScan Systems, INC., Renton, EE. UU.) o Phoenix (Becton Dickinson, Franklin Lakes, EE. UU.), entre otros[3].

A diferencia de otros laboratorios, como los de bioquímica o hematología, que se han beneficiado de la tecnología para simplificar el procesamiento de las muestras y poder obtener de esta manera resultados en un breve espacio de tiempo, la automatización del laboratorio de microbiología es más compleja, dada la gran diversidad de muestras clínicas a analizar y las características inherentes a los diferentes microorganismos.

Los requisitos ideales en una plataforma o equipo de detección e identificación microbiológica serían los siguientes: capacidad de detección de cualquier microorganismo, detección a partir de la muestra clínica directa, obtención de resultados en un periodo corto de tiempo (1-6 h), detección de resistencias, factores de virulencia o toxinas, y una buena relación coste-efectividad.

Recientemente, la espectrometría de masas (EM) ha irrumpido en el laboratorio de microbiología ofreciendo una alternativa rápida y fiable para la identificación de microorganismos. A pesar de que esta tecnología se ha aplicado en diferentes campos durante los últimos años, la reciente creación de plataformas simples y fáciles de utilizar dirigidas al diagnóstico microbiológico ha conllevado su creciente incorporación a los laboratorios de microbiología. Las plataformas comercializadas hasta el momento utilizan la EM para la identificación de microorganismos mediante dos aproximaciones diferentes: la identificación basada en el perfil proteico específico de cada microorganismo (aproximación proteómica) o el análisis de sus ácidos nucleicos (aproximación genómica).

Esta revisión pretende explicar esta tecnología y hacer un resumen de sus aplicaciones actuales o futuras, centrándose en las plataformas comerciales existentes.

Espectrometría de masas

Breve historia

La EM fue concebida hace más de cien años. Sin embargo, no fue hasta la invención de las llamadas técnicas de ionización suave MALDI (por las siglas en inglés de *Matrix-assisted laser desorption/ionization* [desorción/ionización por láser asistida por matriz]) y ESI (por las siglas en inglés de *Electrospray ionization* [ionización por electrospray])[5,6] que esta tecnología empezó a utilizarse en los laboratorios para aplicaciones rutinarias. Estas técnicas de ionización suave permitieron el análisis de proteínas de elevado peso molecular y a la vez facilitaron el análisis de mezclas, ya que mediante estas técnicas los componentes de una mezcla compleja se ionizan sin sufrir apenas fragmentaciones, lo cual simplifica los espectros de masas[7]. En los últimos 20 años, estas nuevas técnicas de EM se convirtieron en indispensables para el avance de la proteómica, sobre todo al ser combinadas con el analizador de masas tipo TOF (por sus siglas en inglés de *Time-of-flight* [tiempo de vuelo]). Aunque la separación de partículas cargadas basada en la masa y el tiempo de vuelo se conoce desde el 1897, la utilización del tiempo de vuelo como medio para obtener un espectro de masas tardó en aplicarse en la EM, y fue en 1946 cuando apareció la primera referencia de su empleo[8].

El MALDI-TOF se utilizaba inicialmente para el análisis cualitativo de biopolímeros, como una herramienta para valorar la pureza de diferentes productos[9]. Debido a las características técnicas de los primeros espectrómetros de tipo MALDI, su utilización para el análisis de analitos de bajo peso molecular resultaba inapropiada debido a que el exceso de matriz enmascaraba la señal de estos. Además, resultaba difícil obtener una aproximación cuantitativa, debido a que la cristalización de la matriz no era uniforme, por lo que se obtenían señales de diferente intensidad según la zona. Actualmente se han subsanado estos inconvenientes y se trata de una técnica ampliamente utilizada para el análisis y la cuantificación de proteínas, péptidos, toxinas, ácidos nucleicos y otros compuestos.

Fundamentos de la tecnología

El espectrómetro de masas produce, separa y detecta iones en fase gaseosa[10]. Los elementos principales que forman un espectrómetro de masas son tres: una fuente de ionización, un analizador de masas y un detector. Los tres elementos se mantienen en una atmósfera de vacío. En la figura 1 se esquematizan los componentes de un espectrómetro de masas.

Fuente de ionización

El resultado de aplicar una fuente de ionización sobre una molécula es la formación de iones, generados por exceso o pérdida de electrones, por lo que dichas moléculas estarán cargadas eléctricamente[8]. En el caso del MALDI, la muestra es embebida en una matriz orgánica, la cual cristaliza en contacto con el aire. Esta mezcla se deposita en una tarjeta de un material conductivo y es irradiada por un láser. La energía del láser causa una desestructuración de la matriz cristalizada generando una nube de partículas. Los iones de dicha nube se extraen al ser sometidos a un campo eléctrico, a través del cual estos son acelerados debido a su carga. Los iones obtenidos son dirigidos hacia el analizador de masas y, posteriormente, al detector. El papel de la matriz es fundamental para los procesos electroquímicos que se producen. Generalmente se trata de sustancias orgánicas que absorben la energía del láser. Algunas de las matrices más utilizadas son el ácido α-ciano-4-hidroxi-trans cinámico, el ácido 2,5-dihidrobenzoico, o el ácido sinapínico.

En el caso del ESI, la muestra se encuentra disuelta en un solvente orgánico y es inyectada a través de un capilar. La diferencia de potencial se genera porque existe un electrodo en contacto con el capilar, mientras que el otro electrodo se sitúa en el detector del espectrómetro. Cuando la muestra sale del capilar se genera una nube de pequeñas gotas cargadas que da lugar a los iones en fase gaseosa. Con esta tecnología, aplicando una diferencia de potencial positiva o negativa se pueden generar cationes o aniones[10].

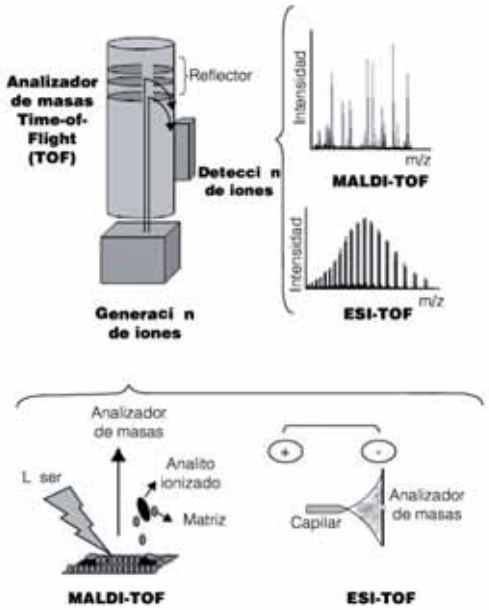

Figura 1. Esquema de un espectrómetro de masas. MALDI: desorción/ionización por láser asistida por matriz; ESI: ionización por electrospray; m/z: ratio masa/carga.

Analizador de masas

Se trata del componente principal del espectrómetro. A pesar de existir diferentes analizadores, el más utilizado en las aplicaciones de la EM dentro del campo de la microbiología es el analizador de tipo TOF. La estructura delimita una zona de vuelo a través de la cual los iones son acelerados adquiriendo una elevada energía cinética, y durante este trayecto se separarán según su ratio masa/carga (m/z). La mayor parte de iones generados poseen una sola carga (z = 1), por lo que la ratio m/z equivale a m. El periodo de tiempo que tarda cada ión en llegar hasta el detector es denominado tiempo de vuelo y depende de dicha ratio[8,11].

Detector

Al final de la zona de vuelo los iones impactan en el detector. En muchos casos, antes del detector encontramos un cuarto elemento, el reflector, cuyo objetivo es compensar las velocidades cinéticas antes de que los iones impacten contra el detector. Con ello se obtiene un aumento en la resolución[12,13]. A partir de la información recogida por el detector, se genera el espectro de masas en el cual se muestra la masa de los diferentes iones de la muestra calculada a partir del tiempo de vuelo. En el eje de las ordenadas del espectro de masas se representan los valores de la ratio m/z, mientras que en el eje de las abscisas se representa la intensidad, es decir, el número de iones de una determinada ratio m/z que ha impactado contra el detector.

En el caso del MALDI-TOF, como se ha comentado con anterioridad, la ratio m/z suele ser equivalente a la masa molecular (m) del analito, por lo que en el espectro típico de MALDI-TOF se observa un solo pico predominante, correspondiente a dicha masa. En el caso del ESI-TOF, en el espectro generado se ven representados todos los iones generados a partir del analito en sus diferentes intensidades, de manera que se observan múltiples picos. En este caso, la masa molecular del analito se obtiene calculando la media de todas las masas moleculares relativas de cada ión obtenido.

Primeras aplicaciones de la espectrometría de masas a la microbiología

Ya en 1975 se publicó un trabajo en que se utilizó una combinación de pirólisis con EM para la identificación directa de microorganismos liofilizados[14]. Mediante los espectros obtenidos pudieron diferenciarse inequívocamente 7 bacterias diferentes (2 grampositivas y 5 gramnegativas). La desventaja de la pirólisis es que el tamaño y el rango de las masas obtenidos por esta técnica son pequeños, por lo cual ofrece poca información estructural. Posteriormente se utilizó otra metodología, el bombardeo con átomos rápidos o FAB-MS (por las siglas en inglés *Fast atom bombardement ionization mass spectrometry*), la cual, a pesar de ofrecer mayor información estructural, solo permite analizar analitos de extractos celulares, como, por ejemplo, lípidos de membrana[15]. Posteriormente, Cain et al.[16] diferenciaron diversas bacterias mediante EM tipo MALDI-TOF con una extracción previa de proteínas hidrosolubles. En 1996 se publicaron los primeros estudios en los que se analizaban células bacterianas intactas sin ninguna extracción previa, sino depositando las células directamente en la tarjeta de un instrumento MALDI-TOF y recubriéndola con una matriz orgánica; Holland et al.[17] generaron los espectros de referencia para 5 bacterias gramnegativas para proceder posteriormente a su identificación a ciegas, pudiendo identificarlas correctamente. En el trabajo de Claydon et al.[18] fueron 10 las bacterias (6 bacterias gramnegativas y 4 bacterias grampositivas) caracterizadas y posteriormente identificadas mediante la tecnología MALDI-TOF.

A partir de estos estudios iniciales, muchos otros utilizaron la EM para resolver problemas microbiológicos (identificación, genotipado, estudio de resistencias, etc.)[19–22]. Poco a poco las bases de datos se fueron ampliando con espectros de los microorganismos más relevantes obtenidos mediante MALDI-TOF.

La espectrometría de masas en el laboratorio de microbiología en la actualidad

Recientemente han aparecido diversas plataformas que han permitido a los laboratorios de microbiología clínica acceder más fácilmente a esta tecnología, ligada en sus inicios a la investigación básica. Desde la comercialización de estas plataformas, el número de publicaciones en las que se describe la utilización de la EM para la identificación de microorganismos ha aumentado de manera exponencial.

Como se detalla a continuación, la EM tiene dos principales aplicaciones: la caracterización de proteínas y de ácidos nucleicos.

Espectrometría de masas dirigida a la detección de proteínas

Actualmente, las dos plataformas comerciales más utilizadas para esta finalidad son MALDI Biotyper (Bruker Daltonics, Leipzig, Alemania) y Axima@SARAMIS (AnagnosTec GmbH, Potsdam, Alemania). Bruker Daltonics ofrece tanto el espectrómetro de masas o hardware, como el software y la base de datos para la interpretación de los resultados (Biotyper 2.0 y Biotyper 3.0 en su nueva versión recientemente disponible). En el caso de Axima@SARAMIS, el espectrómetro de masas y el software de interpretación son fabricados por la compañía Shimadzu (Kioto, Japón), mientras que la base de datos SARAMIS ha sido desarrollada por AnagnosTec GmbH[3,23].

Técnicamente hablando, ambos equipos son muy parecidos. Ambos utilizan la tecnología MALDI-TOF y tienen un rango de detección de proteínas que oscila entre 2.000 y 200.000 Da. La identificación se consigue por la comparación del espectro o perfil proteico obtenido con la base de datos que ofrece cada casa comercial, en la que se encuentran representadas una gran variedad de

bacterias, levaduras y hongos filamentosos. En el caso de la base de datos Biotyper 2.0 se encuentran representados hasta 1.960 microorganismos. Según la información ofrecida por Bruker Daltonics, el sistema MALDI Biotyper permite, además, la identificación a partir de esporas. En el caso de la base de datos SARAMIS, son 1.160 las especies representadas. Ambas bases de datos permiten la incorporación de nuevos espectros, permitiendo personalizarla según las necesidades de cada laboratorio.

Una vez obtenido el espectro de masas y comparado con los existentes en la base de datos, el software le adjudica una identificación y un valor indicativo de la fiabilidad de dicha identificación. En el caso de Biotyper 2.0, este valor se encuentra en un rango que va de 0 a 3, y se recomienda dividir el rango en tres intervalos: un valor ≥ 2 indica una identificación fiable a nivel de especie, un valor entre 1,7 y 2 indica un parentesco muy cercano y ofrece una identificación fiable a nivel de género, y un valor < 1,7 ofrece una identificación poco fiable. En el caso de SARAMIS, el valor adjudicado indicativo de la fiabilidad de la detección tiene un solo punto de corte: un valor superior o igual al 70% indica una identificación con elevada fiabilidad a nivel de especie, mientras que en el caso de obtener valores inferiores se recomienda una segunda evaluación de la muestra[24].

En cuanto a la matriz utilizada, el espectrómetro de masas de Bruker Daltonics utiliza el ácido α-ciano-4-hidroxi-trans cinámico, mientras que en el Axima@SARAMIS se utiliza el ácido 2,5-dihidrobenzoico. En ambos casos se recomienda utilizar 1 µl de matriz para recubrir la muestra.

La tarjeta donde se colocan las muestras en el sistema de Bruker Daltonics consta de 96 pocillos, y éste tarda 1 h en analizar 96 muestras. En el caso de Shimazdu, la tarjeta consta de 48 pocillos y es capaz de realizar 380 identificaciones en 5 h. Ambos sistemas ofrecen la posibilidad de transferir los datos al sistema informático del laboratorio[3].

En cuanto a la muestra de partida, se han descrito dos aproximaciones diferentes: análisis de células intactas o transferencia directa, y extracción previa de proteínas[25]. Los protocolos se describen brevemente a continuación.

- ***Análisis de células intactas.*** Se selecciona la colonia de interés y se aplica directamente sobre la tarjeta del instrumento mediante un asa de siembra o una punta de pipeta. Se necesitan aproximadamente 10^6-10^7 células. A modo de guía, la visualización de la masa microbiana sobre la tarjeta indicaría que la cantidad es suficiente para lograr una identificación. A continuación se cubre la muestra con la solución matriz y se deja secar al aire. Posteriormente se introduce la tarjeta en el instrumento para su análisis. Esta aproximación también puede utilizarse a partir de un cultivo líquido, previa centrifugación para concentrar los microorganismos. El *pellet* obtenido puede ser aplicado directamente a la tarjeta del MALDI.
- ***Extracción de proteínas.*** Tal y como se comenta más adelante, se ha descrito que una extracción previa de proteínas mejora la identificación, sobre todo cuando se trata de bacterias grampositivas y levaduras[26].

Extracción mediante etanol-ácido fórmico. Este protocolo, indicado para el estudio de bacterias no formadoras de esporas, utiliza el etanol para inactivar las bacterias y el ácido fórmico para la disrupción de su pared. Brevemente, el protocolo es el siguiente: se resuspende el *pellet* o colonia de interés con agua bidestilada y etanol. Después de centrifugar y eliminar completamente los restos de etanol, se añade ácido fórmico al 70% y la misma cantidad de acetonitrilo para la disrupción de la pared celular. Una vez centrifugada la muestra, se recoge el sobrenadante y se deposita 1 µl en la tarjeta del instrumento, se recubre con la matriz, se deja secar y se procede a su análisis.

Extracción mediante ácido trifluoroacético (TFA). Este protocolo está indicado para bacterias formadoras de esporas o de elevada patogenicidad. Mediante el TFA se consigue tanto la inactivación de la bacteria como la disrupción de la pared. Brevemente, se resuspende la muestra en TFA al 80% hasta su completa disolución y se deja incubar a temperatura ambiente. Tras añadir agua bidestilada, se le añade un volumen igual de acetonitrilo y se centrifuga. A continuación se deposita 1 µl del sobrenadante en la tarjeta del instrumento, se recubre con la matriz, se deja secar y se procede a su análisis.

A continuación se describen las principales aplicaciones de las plataformas comerciales:

Identificación de microorganismos a partir de colonia.

- ***Bacterias.*** Las dos plataformas anteriormente comentadas ofrecen la posibilidad de identificación directamente a partir de una colonia. Se han publicado diversos trabajos en los que se compara la identificación obtenida por EM, especialmente utilizando el MALDI Biotyper, con los métodos microbiológicos clásicos. Los más relevantes se resumen en la tabla 1. En general se han obtenido valores de concordancia a nivel de especie con los métodos convencionales entre el 84 y el 95%.

  Cherkaoui et al. realizaron un estudio comparando ambas plataformas comerciales, MALDI Biotyper y Axima@SARAMIS[24]. De 720 colonias analizadas, el sistema MALDI Biotyper identificó correctamente y con una elevada confianza el 94,4% de ellas, en comparación al 88,8% obtenido por el sistema Axima@SARAMIS. En las muestras en las que se obtuvo una identificación diferente a la conseguida mediante los métodos convencionales se llevó a cabo la secuenciación del gen 16S para confirmar la identificación. De las identificaciones discrepantes obtenidas utilizando la plataforma MALDI Biotyper, la secuenciación del gen 16S confirmó los resultados obtenidos por dicha plataforma en el 75% de los casos. Este porcentaje fue del 78,3% en el caso de la plataforma de Axima@SARAMIS.

  Además de la identificación a ciegas de aislados clínicos, la EM ha demostrado ser útil a la hora de identificar microorganismos a los que es difícil hacerlo por métodos clásicos, como por ejemplo las bacterias gramnegativas no fermentadoras[30,31] o las diferentes especies del género *Bacteroides*[32]. Así mismo, esta tecnología también permite la identificación y tipificación de *Listeria* spp.[33], *Staphylococcus* spp.[34,35] y *Mycobacterium* spp.[36].

  La mayor limitación de esta técnica es que pueden existir errores en la identificación de bacterias genéticamente muy parecidas, ya que pueden presentar proteomas parecidos. Por ello, el criterio del microbiólogo clínico es crucial en la interpretación de los resultados. La identificación en estos casos puede mejorarse con una extracción proteica previa, pero se recomienda la confirmación por métodos bioquímicos o por secuenciación. Por otro lado, tal y como se explica en el apartado «Espectrometría de masas dirigida a la detección de proteínas», el software adjudica a cada identificación un valor indicativo de su fiabilidad. En el caso de Biotyper 2.0, se ha observado que con valores entre 1,7 y 2, que indicarían un parentesco muy cercano y una identificación fiable tan solo a nivel de género, de hecho se obtienen identificaciones correctas a nivel de especie. Algunos autores consideran estos intervalos demasiado restrictivos a la hora de validar los resultados. Así pues, dentro de dicho intervalo se debería poder adjudicar un elevado grado de fiabilidad a la identificación, sobre todo si se puede disponer de una morfología de colonia que la corrobore[37]. Por último, se ha observado una necesidad de completar la base de datos con más perfiles proteicos para mejorar la identificación correcta a nivel de especie, sobre todo en el caso de bacterias poco comunes[29,38].

Tabla 1
Identificación a partir de colonia con la plataforma MALDI Biotyper (Bruker Daltonics)

Referencia	Tipo de muestra utilizada para el cultivo	Número de colonias analizadas	ID a nivel de especie	ID a nivel de género	Comentarios
Bizzini et al., 2010[26]	Aislados de diversas muestras clínicas (bacterias y levaduras)	1.371 (30 géneros, 60 especies)	Global: 95% GN: 92% GP: 99% LEV: 100%	Global: 98%	En el 22% de los casos fue necesaria una extracción de proteínas previa para obtener una identificación fiable, sobre todo en el caso de levaduras Dificultad en ID bacilos GP ID influida por el medio de cultivo
Seng et al., 2009[27]	Aislados de diversas muestras clínicas[a] (bacterias)	1.660 (45 géneros, 109 especies)	Global: 84%	Global: 94%	Dificultad en la ID de *Propionibacterium acnes*, *Streptococcus pneumoniae*, *Stenotrophomonas maltophilia* y *Shigella* spp.
Van Veen et al., 2010[28]	Aislados de diversas muestras clínicas (bacterias y levaduras)	327 (29 géneros, 80 especies)	Global: 86% GN: 88% CGP: 80% LEV: 95%	Global: 95% GN: 93% CGP: 98% LEV: 100%	Extracción previa de proteínas necesaria para levaduras y, en algunos casos, para bacterias GN no fermentadoras Dificultad ID *Streptococcus* del grupo *viridans*
Sogawa et al., 2011[29]	Aislados de diversas muestras clínicas (bacterias y levaduras)	498 (92 especies)	Global: 91%	Global: 97%	Utilización de 3 espectrómetros de masas con diferente poder de resolución, pero no se encontraron diferencias significantes en la fiabilidad de las identificaciones obtenidas
Marklein et al., 2009[40]	Aislados frescos de muestras clínicas y cultivos tipo (levaduras y hongos)	267 (15 especies de *Candida* spp. y 10 especies de hongos de 7 géneros) y 18 cultivos tipo	Global: 92,5% ID *Candida* spp: 98% ID hongos: 42%	Global: 92,5%	Dificultad de diferenciar *C. dubliniensis* de *C. albicans* Diez de las 17 especies de hongos tuvieron que ser añadidas a la base de datos
Putignani et al., 2010 [41]	Aislados frescos de muestras clínicas (levaduras y hongos)	303 (5 géneros, 19 especies)	Global: 85%	Global: 85%	Conversión de los espectros obtenidos a geles virtuales para investigar la tipificación de las muestras. Diez de los aislados no fueron identificados por no encontrarse en la base de datos

ID: identificación (proporción de resultados obtenidos mediante el sistema BioTyper 2.0 concordantes con la identificación obtenida por los métodos microbiológicos convencionales); GN: bacterias gramnegativas; GP: bacterias grampositivas; CGP: cocos grampositivos; LEV: levaduras.
[a] Sangre, líquido cefalorraquídeo, pus, biopsias, tracto respiratorio, heridas, heces.

- ***Levaduras y hongos filamentosos.*** Dada la gravedad de las fungemias, sobre todo en pacientes inmunodeprimidos, es necesaria una rápida identificación de las mismas. Bader et al.[39] realizaron un estudio en el que compararon ambas plataformas comerciales (MALDI Biotyper y Axima@SARAMIS) para la identificación de levaduras patógenas. Se analizaron un total de 1.192 aislados, que incluyeron 36 especies diferentes. El porcentaje de identificaciones correctas obtenidas mediante la plataforma MALDI Biotyper fue del 97,6%, y en el caso de la plataforma Axima@SARAMIS el porcentaje fue de 96,1%. Los resultados de otros estudios de interés aparecen resumidos en la tabla 1.

Desde nuestro mejor conocimiento, a día de hoy solo se ha publicado un artículo en el que se utiliza una plataforma comercial, la MALDI Biotyper en su versión de software Biotyper 1.0 (Bruker Daltonics), para la identificación de hongos filamentosos del género *Fusarium*[42]. En este estudio se creó una base de datos para la identificación de este patógeno. Se incluyeron 60 cepas obtenidas a partir de aislados clínicos y dos cepas de aislados ambientales. La identificación de todos los aislados fue comprobada mediante secuenciación. Al analizar 49 aislados clínicos a ciegas, se obtuvo una identificación correcta en el 92% de los casos. Cuando no se obtuvo un resultado fiable fue debido a la presencia de especies del género *Fusarium* que no estaban incluidas en la base de datos creada.

A pesar de ser este un territorio poco explorado con la utilización plataformas comerciales, la EM se ha empleado durante años para la identificación de hongos filamentosos. Santos et al.[43] han publicado recientemente una exhaustiva revisión referida al temaen cuestión.

En cuanto a la preparación de la muestra en el caso de las levaduras, en los trabajos revisados (tabla 2) se realiza una extracción de proteínas previa mediante etanol/ácido fórmico. En el caso de hongos filamentosos, Marinach-Patrice et al.[42] realizan una extracción previa mediante ácido trifluoroacético/acetonitrilo. Sin embargo, en su trabajo de revisión Santos et al.[43] comentan que, si bien la lisis era necesaria para la obtención del espectro en los primeros experimentos, en trabajos recientes se ha logrado evitar este paso.

Identificación a partir de hemocultivo. La identificación de bacterias mediante MALDI-TOF directamente a partir de un hemocultivo positivo permite reducir el tiempo de obtención del resultado en comparación con los métodos convencionales, ofreciendo la posibilidad de mejorar el manejo de los pacientes con sepsis. Recientemente se han publicado varios estudios cuyos resultados se resumen en la tabla 2. En la mayoría de los estudios se realizó la extracción previa con etanol/ácido fórmico previamente descrita, ya que como se demuestra en el trabajo publicado por La Scola et al.[44], parece ser más efectiva para las bacterias grampositivas que la extracción mediante TFA. De los 322 hemocultivos analizados mediante la extracción con TFA, en el 59% de las muestras los resultados fueron concordantes con los obtenidos mediante los métodos convencionales. De estos, la concordancia para las bacterias gramnegativas fue del 94%, mientras que para las grampositivas fue del 37%. En una segunda fase del estudio se analizaron un total

Tabla 2
Identificación de bacterias y levaduras a partir de hemocultivos mediante la plataforma MALDI Biotyer (Bruker Daltonics)

Referencia	Número y tipo de muestra analizada	Sistema de hemocultivo	ID a nivel de especie	ID a nivel género	Muestras polimicrobianas	Comentarios
La Scola et al., 2009[44]	240 hemocultivos positivos (bacterias)	BACTEC 9240 (Becton Dickinson)	Global: 76% GP: 64% GN: 87%	Global: 76%	N = 22 ID especie más abundante: 82%	Extracción previa mediante etanol/ácido fórmico Dificultad ID grupo *Streptococcus viridans* y *Streptococcus* spp
Stevenson et al., 2010[45]	179 hemocultivos positivos 33 hemocultivos inoculados (bacterias)	BACTEC 9240 (Becton Dickinson)	Global: 80% GP: 70% GN: 87%	Global: 80%	N = 10 ID especie más abundante: 81%	Extracción previa mediante etanol/ácido fórmico Dificultad ID *Streptococcus mitis* (8 identificados como *S. pneumoniae*, con una fiabilidad* > 1,9) Dificultad ID *Propionibacterium acnes*
Christner et al., 2010[46]	277 hemocultivos positivos (bacterias)	BACTEC 9240 (Becton Dickinson)	Global: 94% GP: 93% GN: 98%	Global: 95%	N = 16 ID especie más abundante: 81%	Extracción previa mediante etanol/ácido fórmico La concentración de microorganismos es un factor crítico para una buena identificación, establecen 10^7 células como concentración mínima
Ferroni et al., 2010[47]	434 hemocultivos positivos (bacterias y levaduras)	BacT/Alert (bioMérieux)	Global: 91% GP: 97% GN: 95%	Global: 96%	N = 15 ID especie más abundante = 87%	Extracción previa mediante etanol/ácido fórmico Dificultad ID *Streptococcus* spp. En las infecciones mixtas, la detección se mejoró si tras la realización de la tinción de Gram se utilizaba una base de datos específica de GP o GN
	312 hemocultivos inoculados (bacterias y levaduras)		Global: 89% LEV: 100%	Global: 95%	–	
Ferreira et al., 2010[48]	318 hemocultivos positivos (bacterias y levaduras)	BACTEC 9240 (Becton Dickinson)	Global: 42% GP: 32% GN: 85%	Global: 72% GP: 65% GN: 98%	No	Extracción previa mediante etanol/ácido fórmico Dificultad de ID *Candida* spp. En bacterias GP, valores entre 1,5–1,7 ofrecieron la misma ID a nivel de género que los métodos convencionales
Moussaoui et al., 2010[49]	503 hemocultivos positivos (532 bacterias)	BACTEC 9240 (Becton Dickinson)	Global: 90% GP: 89% GN: 91%	Global: 90%	N = 21 ID especie más abundante: 81%	Extracción previa mediante etanol/ácido fórmico Dificultad ID *Streptococcus mitis* (identificado como *S. pneumoniae*)
Prod'hom et al., 2010[50]	126 hemocultivos positivos (bacterias)	BACTEC 9240 (Becton Dickinson)	Global: 78% GP: 73% GN: 90%	Global: 79%	No	Extracción previa mediante etanol/ácido fórmico Dificultad ID grupo *Streptococcus mitis* o especies muy cercanas de *Staphylococcus* spp. Dificultad ID bacterias GP o bacterias encapsuladas por propiedades de la pared

ID: identificación (proporción de resultados obtenidos mediante el sistema BioTyper 2.0 concordantes con la identificación obtenida por los métodos microbiológicos convencionales). GN: bacterias gramnegativas; GP: bacterias grampositivas; LEV: levaduras.

* Fiabilidad de la detección según rango de valores: ≥ 2, identificación fiable a nivel de especie; entre 1,7 y 2, identificación fiable a nivel de género; < 1,7 identificación poco fiable.

de 240 hemocultivos y se utilizó el protocolo de extracción con etanol/ácido fórmico, mediante el cual se mejoró la detección de las bacterias grampositivas, tal y como se refleja en la tabla 2. En el trabajo realizado por Ferreira et al.[51] se compararon la identificación a partir de células intactas y la extracción mediante etanol/ácido fórmico. Mediante este segundo método la extracción de proteínas consiguió identificar un mayor número de microorganismos y con valores de fiabilidad más elevados.

En un estudio recientemente publicado, Schmidt et al.[52] compararon los frascos de hemocultivo BD BACTEC Plus-aerobic (Becton Dickinson, Franklin Lakes, EE. UU.), que contienen resinas, con los frascos del sistema BacT/Alert (bioMérieux, Marcy l'Etoile, Francia) SA y FA, de los cuales solo los segundos contienen carbón activo. La plataforma de EM utilizada fue Axima@SARAMIS. Los frascos se inocularon con 103 microorganismos distintos. Para los frascos BD Bactec Plus-aerobic, BacT/Alert SA y BacT/Alert FA la concordancia general con los métodos convencionales fue, respectivamente, del 72, del 46 y del 23%. Para las bacterias gramnegativas la concordancia fue, respectivamente, del 87, del 69 y del 47%. Finalmente, la concordancia en la identificación de las bacterias grampositivas fue, respectivamente, del 60, del 29 y del 5%. Estos datos corroboran lo anteriormente publicado por Szabados et al.[53], quienes demostraron que la sensibilidad de la identificación directa de hemocultivos positivos utilizando el sistema BacT/Alert era baja.

La identificación mediante esta técnica a partir de hemocultivo presenta las mismas limitaciones comentadas anteriormente en «Identificación de microorganismos a partir de colonia».

Identificación de microorganismos a partir de muestra directa. Dado que estas plataformas necesitan una concentración mínima de bacterias para lograr una identificación, resulta difícil la aplicación de la EM a la muestra directa sin pasar por el cultivo. Aun así, sí se han realizado estudios utilizando EM en muestras con una considerable concentración de microorganismos, como es la orina.

Ferreira et al.[54] utilizaron el sistema MALDI Biotyper para la identificación de bacterias directamente a partir de orina mediante la aproximación de células intactas. Sin embargo, en el caso de no obtener una buena identificación, se realizó la extracción previa de proteínas mediante el protocolo de etanol/ácido fórmico. A nivel de especie, la concordancia obtenida con los métodos convencionales fue del 91,8%, y del 92% a nivel de género.

Otras aplicaciones del MALDI-TOF no ofrecidas por las plataformas comerciales. A pesar de no existir aún publicaciones en las que se estudie la resistencia a antibióticos mediante las plataformas comerciales previamente mencionadas, sí se han publicado trabajos en los que se utiliza la EM para la discriminación de cepas de *Staphylococcus aureus* resistentes a la meticilina de aquellas sensibles, ya que presentan variaciones de picos en el espectro de masas[22,55]. De manera similar, en otro trabajo se describe la diferenciación entre cepas de *Escherichia coli* resistentes y sensibles a la ampicilina[56].

Por otro lado, aunque la identificación de virus aún no ha sido puesta a punto por las casas comerciales descritas, también constituye una de las aplicaciones de la EM. Por ejemplo, la identificación de *Norovirus* se ha llevado a cabo a partir de la detección de las proteínas de la cápside[57], y la identificación del virus de Newcastle, por la detección de un polipéptido vírico[58].

Detección de ácidos nucleicos

PCR y MALDI-TOF. SEQUENOM (San Diego, EE. UU.) ofrece un método para la tipificación de bacterias con una precisión comparable al *multilocus sequence typing* (MLST). Esta metodología se basa en una amplificación inicial del DNA de la muestra utilizando un par de *primers* que incorporan en sus extremos 5′ los promotores T7 y SP6, respectivamente, lo que permite la posterior transcripción de ambas cadenas en dos reacciones diferentes. Una vez realizada la transcripción, se utiliza una enzima de restricción para cortar de manera específica el producto amplificado después de cada residuo de uracilo o citosina[59,60]. Estos fragmentos se analizan mediante EM tipo MALDI-TOF. Para la identificación se utiliza una base de datos previamente generada *in silico* de patrones de MLST generados a partir de secuencias de referencia.

Esta tecnología ha demostrado ser útil para la tipificación de *Neisseria meningitidis*[59], para el genotipado de micobacterias[61], así como para el genotipado de los virus de las hepatitis B y C[62,63], entre otros.

PCR y ESI-TOF. Esta tecnología, denominada comercialmente PLEX-ID (anteriormente T5000), ha sido propuesta por Ibis Biosciences Inc. (Carlsbad, EE. UU.), empresa subsidiaria de Abbott Molecular (Chicago, EE. UU.). Se basa en la amplificación de los ácidos nucleicos existentes en la muestra y en el posterior análisis del producto de PCR mediante EM tipo ESI-TOF[64].

El principio básico de esta tecnología es que todos los microorganismos comparten características comunes codificadas en sus genomas, y que estas secuencias conservadas permiten el diseño de *primers* universales. Con ellos es posible amplificar regiones conservadas pero con suficiente variabilidad como para permitir la identificación de los diferentes microorganismos. Además, el uso de distintos pares de *primers* en diferentes pocillos promueve la amplificación de los diferentes microorganismos que puedan coexistir en una muestra clínica sin que haya competencia por los recursos de la PCR. Posteriormente, el producto se analiza por ESI-TOF, determinándose así la masa de los amplicones presentes en la reacción de PCR con tal precisión que proporciona información sobre su composición específica de bases, es decir, en qué proporción se encuentran los diferentes nucleótidos en el amplicón. Sin embargo, no se obtiene la secuencia nucleotídica de los productos de PCR. Esta composición de bases es única para cada gen y específica de cada microorganismo, permitiendo así su identificación gracias al software y a la base de datos proporcionados.

Potencialmente, esta tecnología es capaz de identificar todas las bacterias conocidas, así como las principales familias de hongos patógenos y de virus a partir de cualquier tipo de muestra, previa extracción de los ácidos nucleicos. Además, debido a su diseño, esta técnica también permite la identificación de microorganismos no descritos previamente. Dado que se basa en la amplificación genética a partir de muestra directa, permite además la identificación de microorganismos no cultivables[65]. Otra de las ventajas de esta tecnología es que permite la cuantificación, gracias a un calibrador interno de concentración conocida añadido a cada reacción de PCR, informando del número inicial de genomas del microorganismo identificado en la muestra.

- ***Identificación de bacterias.*** Los *primers* universales más utilizados son los *primers* ribosomales, que amplifican los genes 16S o 23S, pero existen otras regiones ampliamente conservadas, como el gen *rpoB* y otros genes *housekeeping*. El objetivo es utilizar varios pares de *primers*, cada uno dirigido a amplificar un grupo o división de bacterias concreto. De esta manera los diferentes tipos de bacterias que pueda haber en la muestra podrán ser amplificados sin presentar competencia, ya que si la bacteria menos abundante no consigue ser amplificada mediante los *primers* ribosomales, sí podría detectarse mediante los *primers* específicos de su división. El resultado final es la obtención de una mezcla de amplicones que reflejará la complejidad de la muestra inicial. La utilidad de esta tecnología para la vigilancia epidemiológica queda reflejada en diversos trabajos[66–70]. Algunos ejemplos considerados de especial interés se comentan a continuación.

  Identificación de Mycobacterium tuberculosis *multirresistente.* El ensayo molecular descrito por Massire et al.[70] consta de 16 pares de *primers* repartidos en 8 reacciones dúplex. Se analizaron un total de 1.340 aislados, cuya resistencia había sido previamente caracterizada. La sensibilidad y la especificidad en la detección de la resistencia a la isoniazida fueron del 89,3 y del 95,8%, respectivamente. Para la detección de la resistencia a la rifampicina, estas fueron del 96,3 y del 98,6%, respectivamente. Por último, en el caso de la resistencia al etambutol, los valores fueron del 73,6 y del 97,3%. Los valores predictivos positivos y negativos para la detección de estas resistencias fueron, respectivamente, del 97,3 y del 84,3% para la isoniazida; del 96,7 y del 98,5% para la rifampicina, y del 84,4 y del 94,9% para el etambutol.

  El ensayo también demostró su utilidad para la especiación y distinción entre micobacterias pertenecientes al complejo *M. tuberculosis* y micobacterias no tuberculosas (NTM). La sensibilidad en la detección de las bacterias pertenecientes al complejo *M. tuberculosis* fue del 98%, y la especificidad, del 100%. Para las NTM, dichos valores fueron del 97 y del 99,9%. Los valores predictivos positivos y negativos para la identificación de estos dos grupos fueron, respectivamente, del 100 y del 99,5% para el complejo *M. tuberculosis*, y del 99,6 y del 99,6% para las NTM.

  Vigilancia de patógenos respiratorios. En el estudio de Ecker et al.[71] se presentan los resultados obtenidos tras el análisis de diferentes brotes de infecciones respiratorias ocurridos durante los años 2002 y 2003 en diversas instalaciones militares de

Estados Unidos. En el ensayo desarrollado para este fin, se combinaron *primers* universales para la detección de bacterias y *primers* específicos para el genotipado de *Streptococcus pyogenes* basados en el gen *emm*, el cual codifica para la proteína M, un factor de virulencia. En 48 de las 51 muestras analizadas del brote del 2002 se detectó *S. pyogenes* perteneciente al genotipo *emm*3, el cual ha sido asociado a una elevada virulencia. Además, durante el análisis de las muestras se detectó la presencia de otros microorganismos, siendo *Haemophilus influenzae* y *Neisseria meningitidis* los más abundantes, además de *S. pyogenes*, lo cual demuestra que en la mayoría de casos las infecciones respiratorias tienden a ser polimicrobianas. Al analizar muestras de pacientes sanos, el patrón de microorganismos encontrados en la garganta de dichos voluntarios fue diferente, predominando una microbiota de estreptococos del grupo *viridans*. Durante el brote de 2003 se comprobó que la distribución de *S. pyogenes* había cambiado y que el genotipo virulento *emm*3 ya no predominaba.

Identificación de bacterias a partir de hemocultivo. Existen tres trabajos publicados en los que se utiliza esta tecnología para identificar bacterias y levaduras a partir del hemocultivo positivo mediante el ensayo BAC Assay (Abbott Sterile Fluid Bacteria and Candida assay). En dichos trabajos se comparó la identificación obtenida mediante el PLEX-ID con los resultados obtenidos mediante los métodos clásicos: cultivo e identificación mediante perfil bioquímico (Vitek 2 Compact, bioMérieux). En el primero de los dos estudios[72] se obtuvo una concordancia a nivel de género del 94% y a nivel de especie del 93%, de un total de 170 hemocultivos positivos y 199 microorganismos analizados. En el segundo de los trabajos[73] se obtuvo una concordancia a nivel de género del 98,7% y a nivel de especie del 96,5%, de un total de 234 hemocultivos y 211 microorganismos. Finalmente, el último trabajo[74], que se trata de una comparación de esta tecnología con la plataforma comercial de EM por MALDI-TOF MALDI Biotyper, se comenta más adelante. Sin embargo, esta tecnología podría utilizarse a partir de muestra directa; en este caso, la sangre total del paciente antes de inocular el hemocultivo. En el caso de la sepsis, esto permitiría acortar el proceso en una media de 9 h, tiempo necesario para la positivización del hemocultivo, acelerando el proceso diagnóstico y mejorando el manejo de los pacientes. Sin embargo, hasta el momento no existen publicaciones al respecto, aunque nuestro centro está actualmente realizando una evaluación.

- ***Identificación de virus.*** Mediante esta tecnología es posible identificar las principales familias víricas. Se han publicado varios trabajos que demostraban la versatilidad de esta técnica para la detección de virus, como por ejemplo el ensayo diseñado para la detección de los virus de la gripe A y B, con una sensibilidad y una especificidad superiores al 97% (determinadas tras el análisis a ciegas de 656 muestras clínicas)[75]. También se han descrito ensayos para la detección de *Coronavirus*, *Bocavirus* y *Metapneumovirus*, entre otros[75–78].

  Esta tecnología fue una de las primeras en identificar la nueva cepa H1N1 del virus de la gripe responsable de la pandemia del 2009, ya que el ensayo para la identificación de este virus fue capaz de detectar esta nueva cepa sin ninguna modificación previa del mismo[79].
- ***Identificación de factores de virulencia y/o resistencia.*** Incluidos en el ensayo BAC Assay (Abbott Sterile Fluid Bacteria and Candida assay) se encuentran los genes *mecA*, el cual confiere resistencia a la meticilina en cepas de *S. aureus*, la detección de los genes *vanA* y *vanB*, los cuales confieren resistencia a la vancomicina, de especial interés cuando se trata del género *Enterococcus* spp., y la detección del gen *kpc*, de gran relevancia médica en el caso de *Klebsiella pneumoniae* y que le confiere resistencia al carbapenem. La detección de esta última resistencia también ha sido evaluada en *Acinetobacter baumannii*[80]. En el caso de *S. aureus*, también existe la posibilidad de detección de factores de virulencia, como por ejemplo la leucocidina de Panton-Valentine[81,82].

Comparación de las técnicas ESI-TOF y MALDI-TOF para el análisis de hemocultivos. Recientemente se ha publicado un trabajo en el que se comparan estas dos técnicas para la identificación de bacterias y levaduras a partir de un total de 273 hemocultivos positivos[74].

- ***Concordancia entre los resultados obtenidos por EM y los métodos clásicos.*** Ambas técnicas mostraron una muy buena concordancia con la identificación obtenida mediante cultivo y pruebas bioquímicas (Vitek 2, bioMérieux). En el caso del PLEX-ID, la concordancia obtenida fue del 96,7% a nivel de género y del 95,6% a nivel de especie. En el caso del MALDI-TOF, la plataforma utilizada fue la de Bruker Daltonics, y la concordancia obtenida fue del 97,1% a nivel de género y del 94,9% a nivel de especie.
- ***Preparación, análisis y coste por muestra.*** En el caso del PLEX-ID, desde la obtención de la muestra del paciente son necesarias de 4 a 6 h, que incluyen la extracción del DNA, la amplificación por PCR y el análisis de la muestra mediante el espectrómetro de masas. En el caso del sistema MALDI Biotyper, se realizó un subcultivo de los hemocultivos positivos en caldo de enriquecimiento, los cultivos enriquecidos se sembraron en diferentes medios y se utilizó una colonia para el análisis por MALDI-TOF. Como se menciona en «Espectrometría de masas dirigida a la detección de proteínas», esta plataforma permite el análisis de hasta 48 muestras en una hora.

Por otro lado, hay que tener en cuenta que el PLEX-ID necesita diversos reactivos y equipos, tanto para la extracción automatizada de DNA como para la realización de la PCR y el análisis por EM, mientras que para la utilización del MALDI-TOF los requerimientos son mínimos: tan solo es necesaria la matriz orgánica. Por ello, aunque ambas tecnologías requieren de una inversión inicial considerable en instrumentación, el coste por muestra mediante el PLEX-ID se encuentra entre 34 y 70 euros, mientras que el coste por muestra utilizando el MALDI-TOF es de 2 a 4 euros.

Conclusiones

La EM ha demostrado ser una técnica muy útil para la identificación y la tipificación de microorganismos. El reciente desarrollo de plataformas comerciales diseñadas para este fin ha puesto esta tecnología al alcance de los laboratorios de microbiología, con sistemas de fácil manejo y de tamaño y precio asequible. A pesar de necesitar una inversión previa en el instrumento, la rapidez en la obtención de los resultados es un beneficio a tener en cuenta.

La tecnología basada en la identificación del perfil proteico mediante MALDI-TOF ofrece una identificación fiable tanto de bacterias como de levaduras, con el inconveniente de que se necesita una concentración mínima de 10^5-10^6 células para obtener una buena identificación, con lo cual ha sido mayormente aplicado al análisis de cultivos. A pesar de esto, se ha demostrado su utilidad en el análisis directo de orina.

Durante el análisis de hemocultivos, es usual encontrar infecciones polimicrobianas. En estos casos, la tecnología MALDI-TOF generalmente identifica la bacteria más abundante, aunque se ha visto que si se observan manualmente los espectros y el listado de diferentes identificaciones con valores de fiabilidad más bajos, es posible identificar más de un microorganismo. Otra de las desventajas de esta tecnología es la incapacidad actual de detectar resistencias a los antibióticos, salvo las excepciones antes mencionadas.

La plataforma ofrecida por SEQUENOM ofrece una herramienta rápida y fiable de tipificación, pero es necesario conocer

previamente el microorganismo analizado para el diseño de los *primers*. A pesar de resultar una herramienta útil para la epidemiología, no lo es para la identificación de microorganismos de manera rutinaria en el laboratorio de microbiología.

Finalmente, la tecnología PLEX-ID, basada en una PCR y ESI-TOF, permite la detección de cualquier microorganismo. Además, al utilizar *primers* universales no se necesita saber de antemano el microorganismo diana. El mayor potencial de esta tecnología es su capacidad de detección a partir de muestra directa, ofreciendo un resultado en 4 a 6 h, sin ser necesario pasar por el cultivo, aunque de momento son pocos los trabajos en los que se analiza una muestra directa (principalmente, en la detección de virus). Son necesarios, por tanto, estudios clínicos que evalúen la sensibilidad y la especificidad de detección a partir de muestras directas.

A pesar de que la EM ofrece alternativas al cultivo para la identificación de los microorganismos, hay que considerar que, por el momento, aún no existe una tecnología alternativa para el estudio fenotípico de las resistencias y del antibiograma, por lo que de momento el cultivo clásico sigue siendo esencial.

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Conflicto de intereses

Los autores declaran que no existe ningún conflicto de intereses.

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Review Article

Improving the Diagnosis of Bloodstream Infections: PCR Coupled with Mass Spectrometry

Elena Jordana-Lluch,[1] Montserrat Giménez,[1] M. Dolores Quesada,[1] Vicente Ausina,[1,2] and Elisa Martró[1,3]

[1] *Servei de Microbiologia, Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Spain*
[2] *CIBER Enfermedades Respiratorias (CIBERES), 07110 Bunyola, Spain*
[3] *CIBER Epidemiología y Salud Pública (CIBERESP), 08036 Barcelona, Spain*

Correspondence should be addressed to Vicente Ausina; vausina.germanstrias@gencat.cat

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The reference method for the diagnosis of bloodstream infections is blood culture followed by biochemical identification and antibiotic susceptibility testing of the isolated pathogen. This process requires 48 to 72 hours. The rapid administration of the most appropriate antimicrobial treatment is crucial for the survival of septic patients; therefore, a rapid method that enables diagnosis directly from analysis of a blood sample without culture is needed. A recently developed platform that couples broad-range PCR amplification of pathogen DNA with electrospray ionization mass spectrometry (PCR/ESI-MS) has the ability to identify virtually any microorganism from direct clinical specimens. To date, two clinical evaluations of the PCR/ESI-MS technology for the diagnosis of bloodstream infections from whole blood have been published. Here we discuss them and describe recent improvements that result in an enhanced sensitivity. Other commercially available assays for the molecular diagnosis of bloodstream infections from whole blood are also reviewed. The use of highly sensitive molecular diagnostic methods in combination with conventional procedures could substantially improve the management of septic patients.

1. Introduction

Bloodstream infection is a life-threatening condition that results from the presence of microorganisms, generally bacteria or fungi, in the blood [1]. The time window for the administration of an appropriate therapy is less than 6 hours once the symptoms are recognized, and it is optimal to administer broad-range antibiotics within the first hour, preferably after obtaining a blood culture for microbiological diagnosis [2]. Inadequate antimicrobial therapy increases the risk of mortality. Every hour of delay in initiation of appropriate antimicrobial therapy increases the mortality by 7.6% in patients with septic shock [3]. Conventional methods for the microbiological diagnosis of sepsis rely on blood culture followed by biochemical identification. It usually takes 1 to 3 days to obtain both the identification and the antimicrobial susceptibility profile of the pathogen. The major limitation of blood culture methods is that they require a median time-to-positivity of 12 to 17 hours [4]. Another limitation of this method is that the presence of unculturable or fastidious microorganisms may decrease its sensitivity. Culture may also be negative if antimicrobial therapy was begun prior to blood sampling. Thus, there is an urgent need to improve the diagnostic tools for a better management of septic patients.

The ideal diagnostic platform should identify a broad spectrum of pathogens (bacteria, fungi, viruses, and protozoa), determine the susceptibility to a battery of antibiotics, allow the analysis of specimens in high or low throughput, have a low cost per sample, have minimum hands-on time, be user friendly, and, ideally, generate the results in a timely manner for the management of septic patients (6 hours or less). Mass spectrometry technology has recently been introduced in the clinical microbiology laboratory. Using matrix-assisted laser ionization time-of-flight (MALDI-TOF)

spectrometers the diagnostic process may be shortened [5–7] as the identification of the pathogen can be achieved within 30 minutes directly from a positive blood culture [8]. Additionally, this technology is able to detect the resistance to some antibiotics [9], such as the presence of β-lactamases (including carbapenemases), methicillin-resistant *Staphylococcus aureus*, and even vancomycin-resistant *Enterococcus* spp. However, this technology relies on culture and, therefore, a median of 12- to 17-hour delay is unavoidable [4]. In order to further accelerate the diagnostic process, it is desirable to detect and identity pathogens directly from the patient's blood, avoiding the culture step.

Several molecular methods have been developed for the detection of pathogens (mainly bacteria and some fungi) in whole blood. The first assays developed were designed for the detection of a single pathogen of interest and are reviewed by Klouche and Schröder [10]. A single-pathogen approach is not useful for the diagnosis of bloodstream infections, as these infections may be caused by a broad range of microorganisms. This limitation has been overcome in several commercial assays which are able to detect a number of microorganisms [10–12]. These assays are based on two main strategies: the identification of a selected group of pathogens using specific targets (i.e., SeptiFast [13], VYOO [14], and Magicplex [15]) or the detection of a broad range of pathogens using universal/conserved targets (i.e., SepsiTest [16], PCR/ESI-MS [17]).

Use of whole blood in assays designed to detect pathogen nucleic acid is challenging. An excess of human DNA may hamper the detection of pathogen genomic material or may inhibit the PCR reaction [18, 19]; hemoglobin traces may also inhibit PCR-based amplification. Therefore, molecular methods are forced to use a relatively small volume of blood (1 to 5 mL); whereas conventional culture methods use 20–30 mL. This limited volume reduces sensitivity of the molecular methods. Additionally, the bacterial load in adults with bloodstream infection can be as low as 1–10 CFU/mL [20], which may preclude detection of pathogen DNA.

The PCR/ESI-MS technology combines broad-range PCR amplification with the electrospray-ionization time-of-flight mass spectrometry, which is a highly sensitive detection method. Methods have now been developed that allow use of the PCR/ESI-MS technology on whole blood samples, and two clinical evaluations of this system have recently been published [21, 22]. A new version of the instrument is presented that has been designed to improve the sensitivity and implementation in the clinical laboratory. This review describes the current status of the molecular diagnosis of sepsis with emphasis on the PCR/ESI-MS technology.

2. Summary of Commercially Available Molecular Assays for the Diagnosis of Bloodstream Infections from Whole Blood

2.1. SeptiFast (Roche, Mannheim, Germany). SeptiFast is a multiplex real-time PCR assay that detects 25 pathogens including five *Candida* species and *Aspergillus fumigatus* [13]. The presence of the resistance gene *mec*A may be detected with a separate test. The initial volume of blood required is 3 mL (using the manual DNA extraction protocol 1.5 mL aliquots are processed in duplicate) or 1.5 mL (using the automated DNA extraction) [23]. The region amplified in this assay is the internal transcribed spacer region (IST), which is located between the 16S and 23S ribosomal genes for bacteria and between 18S and 5.8S ribosomal genes for fungi [13]. The amplification is performed with a LightCycler 2.0 instrument; different pathogens are detected through specific fluorescent probes. The time-to-result using this approach is 4.5–6 hours. This assay has been widely evaluated in the clinical setting; however, the results are conflicting with reported sensitivities ranging from 15% to 98% in ICU patients [24]. Recently, Chang et al. reviewed all the available literature reporting use of the SeptiFast assay and performed a meta-analysis that included data on 6,012 patients from 35 selected studies. The overall calculated sensitivity of SeptiFast was 75.0% (95% confidence interval, 65.0–83.0%), and the specificity was 92.0% (95% confidence interval, 90.0–95.0%). The performance of the test clearly varies depending on the group of patients tested.

2.2. SepsiTest (Molzym, Bremen, Germany). The SepsiTest assay is based on broad-range PCR amplification followed by sequencing. In the SepsiTest two 1 mL aliquots of blood are processed in duplicate and human DNA is selectively degraded prior to the bacterial cell lysis step [16]. Several studies using this approach for the diagnosis of sepsis have been published. The largest study ($N = 342$) [16] reported a sensitivity and specificity of 87.0% and 85.8%. Two smaller studies reported lower values of sensitivity of 46.0% ($N = 50$) [25] and 37.5% ($N = 75$) [26]; specificities were 100% [25] and 86.6% [26].

2.3. VYOO (SIRS-Lab, Jena, Germany). The VYOO assay is a multiplexed PCR analysis that detects 34 pathogens, including six species of *Candida* and *Aspergillus fumigatus*, as well as several resistance genes (methicillin resistance gene *mec*A, vancomycin resistance genes *van*A and *van*B, and β-lactamase genes *bla*SHV and *bla*CTX-M). The amplified products are visualized using a conventional gel electrophoresis, and the time-to-result is 8 hours. For this assay, microbial DNA from 5 mL of blood is enriched: total DNA is applied to an affinity chromatographic column that specifically binds the microbial DNA (LOOXTER) [27]. Additionally, human DNA is depleted during the extraction step. This assay has a sensitivity ranging from 38.0% to 60.0% [14, 25, 28].

2.4. Magicplex Sepsis Real-Time Test (Seegene, Seoul, Korea). In the Magicplex Sepsis assay, three PCR reactions are necessary to achieve the identification at the species level of the pathogen. First, a conventional PCR amplification step is performed. In this step, primers designed to amplify genomic material from 91 microorganisms (85 bacteria, five species of *Candida*, and *Aspergillus fumigatus*) and three resistance genes (methicillin resistance gene *mec*A and vancomycin resistance genes *van*A and *van*B) are used. A real-time PCR is then carried out in a screening step for identification of the

group or genera level of pathogens present. Finally, a second real-time PCR is performed to achieve the identification at species level. Identification of 21 bacterial species, five *Candida* species, and *Aspergillus fumigatus* is possible. For the DNA extraction, 1 mL of whole blood is used and human DNA is removed prior to the lysis of microorganisms. The time-to-result of this assay is 6 hours. To our knowledge, only one study using this approach for the molecular diagnosis of sepsis has been published [15]. The sensitivity and specificity were reported to be 65.0% and 92.0%, respectively.

3. The PCR/ESI-MS Technology

3.1. Principles of the Technology. This technology combines broad-range PCR with ESI-MS mass spectrometry. Briefly, after the PCR, amplicons are desalted and analyzed by mass spectrometry. ESI-MS is used to determine the molecular mass of each amplicon, which is then used to calculate the base composition of each amplicon. The base compositions of multiple amplicons from different regions of the genome are compared to an extensive database and the identification of the pathogen is achieved (Figure 1). Even though the base composition analysis is not as informative as sequencing, it has enough discrimination power for the detection and identification of hundreds of microbial pathogens. A broad bacteria and *Candida* detection assay (BAC assay; Ibis Biosciences, an Abbott company, Carlsbad, CA, USA) has been designed for use in clinical research to identify more than 600 bacteria and *Candida* species. The BAC assay also detects resistance genes for three clinically relevant antibiotics: methicillin (*mec*A), vancomycin (*van*A and *van*B), and carbapenem (*blaKPC*).

3.2. PCR Amplification. The amplification of conserved regions of the genome has been widely used for the identification of microorganisms at the species level. Although the most common targets are the ribosomal DNA genes (i.e., 16S for bacteria and 18S for fungi), several housekeeping genes (i.e., *tufB*, *rplB*, *valS*, and *rpoB*) are also useful for the identification of pathogens [10, 11, 29]. Within these genes, highly conserved regions are used as priming sites, but the region amplified contains enough variability for the discrimination between species. For instance, in order to identify bacterial and *Candida* species, the BAC assay includes thirteen pairs of primers targeting different conserved regions (nine primers pairs for bacteria and four for *Candida* species). An advantage of using PCR primers designed for several conserved regions with varying degrees of specificity is that when more than one microorganism is present, there is redundancy of coverage across various primer pairs. This is especially relevant when the different microorganisms are present in different abundances, as using several nonoverlapping primer pairs may allow amplification of the less abundant species. Redundant amplification also prevents missed detections due to mismatches in single priming sites [29, 30].

3.3. Detection and Quantification of PCR Products. Mass spectrometry is highly sensitive and can detect small amounts of a nucleic acid of a given sequence even in a complex mixture. The PCR/ESI-MS system employs a software algorithm that calculates a base composition for each amplicon based on mass, compares these to an extensive database, and achieves the identification of the pathogen [17, 31].

Another feature of this technology is that it allows a relative quantification of the microorganism present in the specimen. This is achieved by the use of an internal standard that is amplified with the same primer pairs as those for amplification of the target gene. The internal standard has a different base composition and thus can be differentiated. As this synthetic standard is added to each PCR well at a known copy number, the comparison between standard and microbial DNA permits quantification. In the absence of a PCR product, the internal standard serves as PCR positive control to exclude PCR inhibition.

3.4. Usefulness of the PCR/ESI-MS for the Diagnosis of Bloodstream Infections. The accuracy of BAC assay for the diagnosis of bloodstream infections was first evaluated on blood culture specimens [32–34]. Those studies demonstrated robustness of the technology in terms of accuracy of the identifications. However, with the introduction of MALDI-TOF instruments for the identification of pathogens from positive blood culture based on their protein/peptide profile, it became clear that PCR/ESI-MS would not be able to compete on either a time-to-result or cost-per-sample basis with MALDI-TOF [35].

An advantage of the PCR/ESI-MS assay relative to the MALDI-TOF assay is that PCR/ESI-MS has been optimized to achieve a rapid diagnosis from direct clinical specimens. To date, two clinical evaluations of the PCR/ESI-MS for the diagnosis of bloodstream infections from whole blood have been published. Jordana-Lluch et al. [21] evaluated this system analyzing 247 whole blood specimens (75 with a paired positive blood culture and 172 with a negative blood culture result), and Laffler et al. [22] tested 464 whole blood specimens with a positive paired blood culture and 442 with a negative blood culture result. The agreement between blood culture followed by biochemical identification and PCR/ESI-MS was good in both studies: 77.1% in the Jordana-Lluch et al. study [21] and 78.6% in the Laffler et al. study [22].

Polymicrobial infections were detected in both studies by conventional and/or molecular methods. The agreement between methods on these specimens was low, as most of the mixed infections were detected by only one of the two methods. However, the use of this molecular method in addition to blood culture would have resulted in additional detections of clinically relevant microorganisms in some cases, which could have influenced patient outcome.

In a number of cases in both studies, PCR/ESI-MS detected microorganisms in whole blood specimens with a paired negative blood culture. The clinical relevance of the additionally detected microorganisms was investigated through clinical records review in order to discriminate between probable contaminants and true pathogens. The proportions of detected microorganisms with clinical significance not isolated by conventional methods were 7.5% (13 out

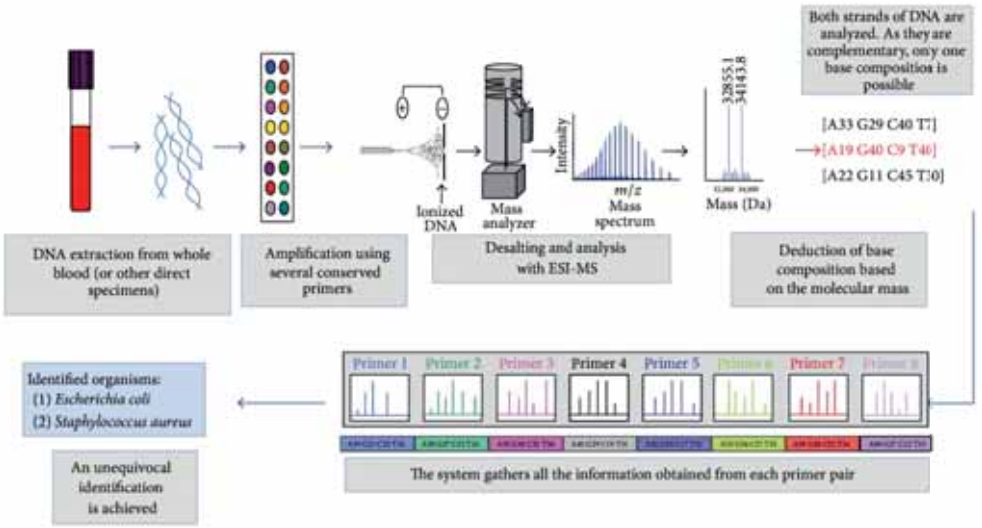

FIGURE 1: Schematic workflow of PCR/ESI-MS system. ESI-MS: electrospray ionization mass spectrometry. Part of the figure has been modified from Ibis Biosciences, a subsidiary of Abbott Molecular, with permission.

172 blood culture negative cases) [21] and 7.2% (31 out of 431 blood culture negative cases) [22]. These findings are highly relevant, as conventional methods were not able to diagnose the etiology of infection in the culture-negative patients.

The sensitivity of the system was calculated using different approaches in each study. Jordana-Lluch et al. disregarded those specimens with a polymicrobial identification by either or both methods, as the events with one correct detection but with a disagreement in the second one were difficult to catalogue as "true positive" or "false positive." In those terms, the sensitivity of the PCR/ESI-MS was 50.0%. Laffler et al. performed a theoretical approximation of the sensitivity based on the historical blood culture positivity rate in their center. They extrapolated the experimentally obtained PCR/ESI-MS positivity rate in order to obtain the number of negative blood cultures that, if processed by the PCR/ESI-MS, would have additionally tested positive. The estimated sensitivity of PCR/ESI-MS using this theoretical approach was 85.9%. This extrapolation may have led to a biased estimation of the sensitivity.

Although these sensitivity values are not directly comparable because they were calculated in different ways, the Laffler et al. study had a higher detection rate of the PCR/ESI-MS on whole blood specimens with a paired positive blood culture. As many factors may affect the sensitivity of molecular methods, a direct comparison between studies is difficult. Differences in the clinical condition of the patients, their characteristics (e.g., age, antimicrobial treatment at the time of the blood draw), the microorganisms isolated, the number of blood cultures taken, and the volume of blood drawn for culture may result in differences between studies [36]. The limitations in sensitivity of the evaluated version of the PCR/ESI-MS technology result from the amount of blood tested in comparison with the blood culture (1.25 versus 20–30 mL). This problem has been overcome with the new version of the PCR/ESI-MS technology, which uses higher volumes of whole blood reducing the limit of detection 4-5-fold.

3.5. The New Version of PCR/ESI-MS. Since its original description by the team of Ibis Biosciences, the PCR/ESI-MS technology has been continuously evolving. The first instrument, named TIGER (for Triangulation Identification for the Genetic Evaluation of Risk) [31], was initially designed for biodefense and surveillance applications, due to its capability to identify previously unknown and unculturable microorganisms. Shortly after, a commercial version of this technology appeared, the Ibis T5000 [17, 30]. In this format, the sample processing was automated and a software system permitted management of the instrumentation, signal analysis, and report generation. This version of the instrument was intended to be used in health and industry settings; it provided highly sensitive detection without the need for a highly trained operator. With the incorporation of Ibis Biosciences into the Abbott group, the system was upgraded [29]. This system, the PLEX-ID, was used in the aforementioned studies [21, 22, 32, 33, 35]. Recently, a newer version has been developed with improvements focused on the analysis of direct patient specimens. One of the principal changes is the use of a larger volume of blood (5 mL) in order to increase sensitivity. Changes in the extraction process allow

Table 1: Comparison between PLEX-ID and the new version of PCR/ESI-MS.

	PLEX-ID	New version
Volume of whole blood analyzed	1.25 mL	5 mL
Samples per run of nucleic acid extraction	1–24 (24-well plate format, manual dispensation of reagents and specimens)	1–6 (ready-to-use individual reagent cartridges)
Minimum number of samples during MS analysis	6 (96-well plate)	1 (one individual 16-well strip per specimen)
Preanalytical analysis equipment	4 (mechanical lysis, magnetic nucleic acid extraction, fluid handler, and thermocycler)	3 (mechanical lysis, magnetic nucleic acid extraction, and thermocycler)
Analytic equipment	1 large instrument (desalting and MS in the same instrument)	2 bench-top instruments (separation of desalting and MS)
Time-to-result	6 h	5-6 h

ESI-MS: electrospray ionization mass spectrometry.

the use of several types of primary tubes and extraction protocols are tailored to the needs of the clinical laboratory. Another important improvement is that one to six specimens can be analyzed at a time. Finally, the mass spectrometer is a bench-top instrument, facilitating installation in clinical laboratories. In Table 1, a comparison between the PLEX-ID and the new version of the PCR/ESI-MS technology is depicted. A preliminary evaluation of this new version has shown a better sensitivity in the detection of pathogens in direct clinical specimens. Further evaluations are currently underway.

3.6. Other Applications in the Clinical Diagnosis of Infectious Diseases. The versatility of the PCR/ESI-MS has been widely demonstrated. In 2012, Wolk et al. [37] reviewed the existing literature of this technology. In this section, we aim to summarize its potential applications in the clinical laboratory as well as to point out several new publications not included in the previous review.

A PCR/ESI-MS assay is able to differentiate species in the *Mycobacterium tuberculosis* complex and classify these species based on drug resistance [38, 39]. This technology has also proved its usefulness for epidemiological proposes, given that it enables molecular genotyping [40]. For instance, genotyping of *Staphylococcus aureus* [41, 42], *Acinetobacter baumannii* [43–45], and respiratory pathogens [46, 47] has been performed in a variety of clinical settings. Bhatia et al. [48] used PCR/ESI-MS to identify a *Streptococcus intermedius* species from cerebrospinal fluid (CFS) and from a fixed biopsy in a patient with a central nervous system (CNS) infection. Although this infection had a respiratory origin, both bronchoalveolar lavage and CFS cultures were negative. Farrell et al. [49] investigated the capability of PCR/ESI-MS to identify pathogens on several specimens collected from patients undergoing antimicrobial treatment. A total of 76 clinical specimens including swabs, blood cultures, fluids, and tissues were collected from 47 patients. From those, 72% (55/76) were culture negative, whereas 76% (58/76) were PCR/ESI-MS positive.

Major viral families can also be detected using this approach. Of special interest is the new version of the Viral IC assay designed for the diagnosis of opportunistic viral infections of immunocompromised patients by viruses such as *Herpesvirus*, *Adenovirus*, *Parvovirus*, *Picornavirus*, and *Polyomavirus*. The ability of the assays on the PCR/ESI-MS system to detect influenza virus, coronavirus, respiratory syncytial virus, human adenovirus, human metapneumovirus, vector-borne flaviviruses, and alphaviruses has been demonstrated [50–52]. Moreover, this technology shows a great promise for the global surveillance of influenza virus [53–55]. Remarkably, it was able to detect the novel H1N1 strain during the 2009 influenza virus outbreak without any modification in the Influenza Surveillance Assay (Ibis Biosciences, Carlsbad, CA, USA) [56].

Fungi are causative agents of infections, but due to the slow growth of these microorganisms, identification by culture is often impractical. Recently, a new assay for the PCR/ESI-MS systems has been validated for detection of *Aspergillus* spp., *Candida* spp., *Pneumocystis* spp., *Cryptococcus* spp., *Mucor* spp., and *Rhizopus* spp. [57]. Concordance rates between PCR/ESI-MS and phenotypic identification and sequencing were 89.7% at the genus level and 87.4% at the species level. Although most of the experiments in this study were performed with reference strains and clinical isolates, detection of *Aspergillus terreus* directly from a culture-negative bronchioalveolar lavage was demonstrated [58].

4. Conclusions

Microbiological diagnosis has historically relied on culture. Isolation of the causal agent provides an irrefutable proof of an infection and allows pathogen identification and determination of antibiotic susceptibility. However, many microorganisms are unculturable, fastidious, or slow-growing. Additionally, prior antimicrobial treatment negatively affects culture-based tests. In the case of bloodstream infections, lack of detection is critical. A significant percentage of blood cultures are negative despite the high likelihood of a bacterial or fungal infection [2]. Lack of culturability and the time to answer mean that many septic patients are not appropriately treated. PCR/ESI-MS is a robust technology that offers a rapid alternative for the diagnosis of bloodstream as well as

other infections. Although being not currently commercially available, the new presentation of the technology has been improved in several aspects that significantly enhance sensitivity. The main advantage of this technology is that it can be used on direct patient specimens, avoiding the culture step. Using this technology as a complement to conventional methods will offer a real improvement in the management of septic and other critically ill patients (i.e., patients suffering from meningitis or fever of unknown origin). Its versatility for the detection of different kinds of microorganisms will make this technology a highly valuable tool in the clinical laboratory.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Appendix III: Posters

10-GMA-2897-ASM. Identification of Bacteria and Fungi from Blood Culture Bottles using the Ibis PLEX-ID

110th General Meeting of rhe American Society of Microbiology, May, 23rd-27th, 2010, San Diego, CA (United States). Poster (Abstract no. C-1119/231).

E. Jordana-Lluch[1], E. Martro[1], S. Molinos[1], M. Gimenez[1], J. Mòdol[2], L. L. Cummins[3], L. B. Blyn[3], R. Sampath[3], D. J. Ecker[3], V. Ausina[1].

1. Microbiology Deptarttment, Hospital Universitari Germans Trias i Pujol, Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, Badalona, SPAIN. 2. Unitat de Curta Estada, Emergency Unit, Hospital Universitari Germans Trias i Pujol, Department of Genetics and Microbiology, Universitat Autònoma de Barcelona, Badalona, SPAIN. 3. Ibis Biosciences, Inc.,Carlsbad, CA.

Background: Achieving a rapid etiologic diagnosis of bacteremia is crucial for the establishment of an appropriate antibiotic treatment and patient survival. The gold standard in identification of the causal agents is blood bottle culturing followed by classical microbiological identification. These methods are slow and a more rapid diagnosis would result in better patient management. Molecular methods offer a rapid alternative for microorganism identification from positive blood cultures. We used the BAC Spectrum BC Assay to identify the organisms in positive blood culture bottles from patients with bacteremia and compared the results to traditional microbiological identification.

Methods: In this study, the Abbott PLEX-ID (Ibis Biosciences and Abbott Molecular, Inc.) was used to analyze the DNA of 168 blood cultures (138 positive and 30 negative). The identification of the causative agents was previously achieved by standard culture methodology. The DNA of the blood cultures was extracted with the KingFisher 96 (Thermo Scientific), or by a manual crude lysis method. All the eluates were amplified in 96-well plates using the BAC Spectrum BC Assay and analyzed with the PLEX-ID system.

Results: In 124 of the 168 blood cultures (83 %) the correct identification was achieved at the genus/species level. In 17 additional cases (10 %) the genus was correctly identified, for a total of 93 % correct identification at the genus level. In 3 cases (2 %) the PLEX-ID system detected a microorganism that was not found by standard methods. Multiple infections were identified in 15 samples by culture, 11 of which were also identified correctly by the PLEX-ID. Discrepant microorganisms were reported by the PLEX-ID in 6 of the 168 blood cultura identifications.

Conclusions: The PLEX-ID system is a promising technology which permits a rapid and reliable identification of microorganisms from automated blood culture.

Paper #
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Identification of Bacteria and Fungi from Blood Culture Bottles using the PLEX-ID System

E. Jordana Lluch[1], E. Martró[1], S. Molinos[1], M. Giménez[1], J. Mosqué[2], L. L. Cummins[3], L. B. Blyn[3], R. Sampath[3], D. J. Ecker[3], V. Ausina[1]
1.Microbiol. Dept., Hosp. Univ.Germans Trias i Pujol, Dept. of Genetics and Microbiol., Univ. Autònoma de Barcelona, Badalona, SPAIN. 2. Unitat de Curta Estada, Emergency Unit, Hosp. Univ. Germans Trias i Pujol, Badalona, SPAIN. 3. Ibis Biosci., Inc, Carlsbad, CA.

Contact Information:
viaduna.germanstrias@gencat.cat
Phone: (+34) 934657804
Fax: (+34) 934915883

Introduction

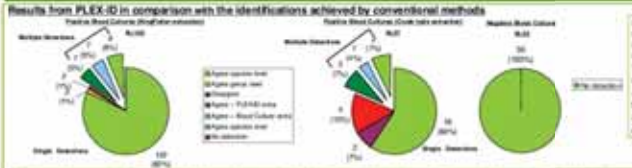
Achieving a rapid etiologic diagnosis of bacteremia is crucial for the establishment of an appropriate antibiotic treatment and patient survival. The gold standard in identification of the causal agents is blood bottle culturing followed by classical microbiological identification. These methods are slow and a more rapid diagnosis would result in better patient management. Molecular methods offer a rapid alternative for micro-organism identification from positive blood cultures.

Materials & Methods

In this study, the Abbott PLEX-ID (Ibis Biosciences and Abbott Molecular, Inc.) was used to analyze the DNA of 205 blood cultures (176 positive and 30 negative). The identification of the causative agents was previously achieved by standard culture methodology[1]. The DNA of the blood cultures was extracted with the KingFisher 96 (Thermo Scientific), or by a manual crude lysis method. All the studies were amplified in 96-well plates using the MAC Spectrum EC Assay and analyzed with the PLEX-ID system.

Results

Results from PLEX-ID in comparison with the identifications achieved by conventional methods



In 1 of the 7 samples where not all microorganisms were detected, the PLEX-ID correctly identified all of them, but 1 microorganism was under the reportable threshold (<500 genomes/well), therefore it was reported as Agree + Blood culture extra.
In the samples where the DNA was obtained through the crude lysis method, 0.5 mL of the blood culture was used instead of 1.1 mL (King Fisher method). Even so, an identification was achieved in all but 4 cases. In 2 samples the correct ID was achieved and, although it was under the detection threshold (500 genomes/well), they were classified as Agree species level.

Clinical records were reviewed for patients that showed discrepant results by the two methods. In 4 cases the clinical records pointed to a clinical significance for the microorganisms detected (Table 4). In other cases, an extra microorganism was detected by the PLEX ID, and were classified as contaminants. *Streptococcus* spp. (1), *Lactobacillus* spp. (1), coagulase negative *Staphylococcus* (1). In 2 cases the PLEX-ID detected microorganisms that are not common contaminants, but no clinical significance was found according to clinical records: *Pasteurella multocida* (1), *Streptococcu pneumoniae* (1). Finally, 2 samples were from patients who had bacteremia due two microorganisms but they grew in different blood culture bottles. The PLEX-ID detected both of them in the two DNA eluates for those patients.

Conclusions

The PLEX-ID system is a promising technology which permits a rapid and reliable identification of microorganisms after automated blood culture. Therefore, the identification of the pathogens from positive blood cultures can be obtained in a maximum of 6 hours instead the 24-48 hours using the conventional identification methods. An early correct antibiotic therapy would decrease the morbimortality of sepsis.

References

1. Ecker DJ *et al.* Ibis T5000: a universal biosensor approach for microbiology. Nat Rev Microbiol. 2008;6: 553-558.
2. Mancini N. *et al.* Clin Microbiol Rev. 2010; 23(1):235-51

Acknowledgments

All reagents were supplied by IBIS Biosciences.
Financial support was provided by the Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica.

136. Diseno y optimizacion de un sistema de deteccion molecular por microarrays para la rapida identificacion de bacterias grampositivas y hongos en hemocultivos positivos.

XV Congreso Nacional de la Sociedad de Enfermedades Infecciosas y Microbiología Clínica (SEIMC), 1-4 Junio, 2011, Málaga. Poster (N.º 136).

A.I. Moraga Quintanilla[1], O. Salazar Torres[1], **E. Jordana Lluch[2],** E. Martró Català[2], S. Molinos Abós[2], M. Giménez Pérez[2], R. Cospedal García[1], V. Ausina Ruiz[2] y M.L. Villahermosa Jaén[1]

1. GENOMICA S.A.U. Coslada. 2. Hospital Universitari Germans Trias i Pujol. Badalona.

Objetivos: Diseño y optimización de un método de identificación rápida a nivel de género y especie de bacterias grampositivas y hongos a partir de hemocultivos positivos.

Métodos: El DNA bacteriano fue extraído de manera automática a partir de hemocultivo positivo usando el extractor automático Easymag de bioMérieux. Se ha optimizado una multiplex-PCR a partir de cebadores específicos para cada taxón diseñados en regiones constitutivas del genoma bacteriano y fúngico. Los productos amplificados fueron marcados para su posterior hibridación en una plataforma de microarrays de baja densidad (CLART-Strip®). Las sondas específicas para cada taxón, e inmovilizadas en el microarray fueron diseñadas a partir de regiones flanqueantes a la zona de hibridación de los cebadores. Las hibridaciones específicas fueron realizadas en formato de tiras de 8 pocillos con el microarray impreso en el fondo del pocillo. Se han incluido cebadores y sondas específicas para la detección específica del gen *mec*A, responsable de la resistencia a meticilina en *Staphylococcus aureus.* Se ha incluido el análisis automático de datos mediante el diseño de una aplicación informática específica.

Resultados: El ensayo detecta correctamente 14 especies de bacterias grampositivas pertenecientes a los géneros *Streptococcus* (*S. pyogenes, S. dysgalactiae, S. pneumoniae, S. agalactiae, S. mitis, S. sanguinis, S. parasanguinis, Streptococcus del grupo milleri (S. anginosus, S. constellatus) y Streptococcus* spp.) *Staphylococcus (S. aureus, S. epidermidis, S. haemolyticus, S. hominis* [incluyendo la deteccion de *mec*A]), *Clostridium perfringens, Listeria monocytogenes, Enterococcus (E. faecium, E. faecalis)*, y 3 especies del género *Candida* (*C. albicans, C. glabrata, C. krusei,* incluyendo la detección genérica de *Candida* spp, y hongos en general). El ensayo ha proporcionado una sensibilidad y especificidad analítica de un 100 % y una sensibilidad y especificidad diagnóstica superior al 95 % demostrada tras el análisis de 305 muestras, identificando correctamente el 94 % de las mismas.

Conclusiones: Los resultados de identificación se obtuvieron 4 horas después de la detección del hemocultivo como positivo. El ensayo permite el procesamiento de hasta 96 arrays en una misma tanda, siento la unidad mínima de ensayo la tira de 8 arrays, y propor cionando una identificación rápida y fiable, la cual permitirá la aplicación de un tratamiento más dirigido a nivel de terapia antimicrobiana en los estadios tempranos de la infección.

Diseño y optimización de un sistema de detección molecular por microarrays para la rápida identificación de bacterias grampositivas y hongos en hemocultivos positivos.

Ana Isabel Moraga[a], Oscar Salazar[a], Elena Jordana[a], Elisa Martró[b], Sònia Molinos[b],Montserrat Giménez [b], Rosario Cospedal[b], Vicente Ausina[b] y M. Luisa Villahermosa[a].

[a]GENOMICA S.A.U. Alcarria 7. Pol. Ind. Coslada, 28823 Coslada, Madrid.

[b]Servei de Microbiologia, Hospital Universitari Germans Trias i Pujol,08916 Badalona, Barcelona.

CLART® SeptiBac+

INTRODUCCION

La sepsis está reconocida como una de las principales causas de mortalidad y morbilidad en los hospitales y es provocada principalmente por bacterias y hongos. Los hospitales suelen tardar un mínimo de 24 horas en identificar los patógenos bacterianos/fúngicos, así como las reacciones asociadas a los fármacos a partir de los cultivos de sangre. La detección precoz de los patógenos mediante herramientas de diagnóstico molecular puede facilitar el diagnóstico rápido de la bacteriemia/fungemia, y la rápida administración de una terapia antibiótica adecuada, además de reducir el abuso inadecuado de antibióticos. Debido a que existe una gran variedad de patógenos bacterianos asociados a esta entidad, se hace necesario el diseño y optimización de herramientas de diagnóstico rápido, precisas y fiables para su detección. Se ha diseñado y optimizado un sistema rápido de detección e identificación por múltiplex-PCR y posterior hibridación en microarrays de baja densidad.

OBJETIVOS

Desarrollo y validación de un kit de diagnóstico molecular basado en multiplex-PCR e hibridación en arrays con sondas de ADN para la identificación y caracterización de patógenos en pacientes con sepsis.

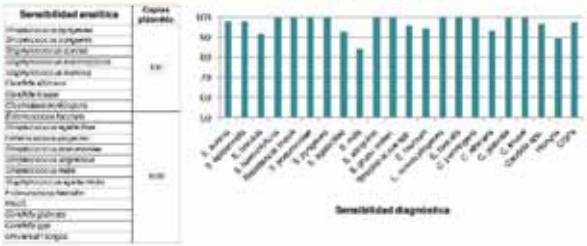
METODOS

El ADN se obtuvo con el extractor automático NucliSens Easymag (BioMérieux) a partir de hemocultivos. Se ha optimizado una multiplex-PCR a partir de cebadores específicos para cada taxón diseñados en regiones constitutivas del genoma bacteriano y fúngico. Los productos amplificados fueron marcados para su posterior hibridación en una plataforma de microarrays de baja densidad (CLART-Strip®).

Las sondas específicas para cada taxón, inmovilizadas en el *microarray*, se diseñaron a partir de regiones que flanquean la zona de hibridación de los cebadores. Las hibridaciones específicas se realizaron en formato de tiras de 8 pocillos (CLART-Strip®) con el *microarray* impreso en el fondo del pocillo. Los resultados se compararon con los obtenidos con el cultivo convencional.

RESULTADOS

El ensayo detecta correctamente 14 especies de bacterias grampositivas pertenecientes a los géneros *Streptococcus* (*S. pyogenes, S. dysgalactiae, S. pneumoniae, S. agalactiae, S. mitis, S. sanguinis, S. parasanguinis, Streptococcus* del grupo *milleri (S. anginosus, S. constellatus)* y *Streptococcus* spp.) *Staphylococcus* (*S. aureus, S. epidermidis, S. haemolyticus, S. hominis* (incluyendo la deteccion de *mecA*)), *Clostridium perfringens, Listeria monocytogenes, Enterococcus* (*E. faecium; E. faecalis*), y 3 especies del género *Candida* (*C. albicans, C. glabrata, C. krusei*, incluyendo la detección genérica de *Candida* spp, y hongos en general).



Tras el análisis de **596 muestras** se obtuvieron los siguientes resultados:

- Sensibilidad = 97%
- Especificidad = 100%
- Reproducibilidad = 95.4%
- Repetibilidad= 96.9%

CONCLUSIONES:

•Se ha desarrollado un ensayo sensible ofreciendo unos valores de sensibilidad superiores al 95%.

•El ensayo es fácilmente estandarizable en un laboratorio clínico y proporciona los resultados en 4 horas.

•El ensayo es multiparamétrico, permitiendo la detección simultánea de un gran número de patógenos, aportando una alta robustez, con repetibilidad y reproducibilidad superiores al 95%.

1.21. Design and optimization of micro array based in Vitro assay, CLART®SeptiBac+, for the rapid identification of gram positive bacteria and fungi present in positive blood cultures.

European Meeting on Molecular Diagnostics, October, 12th-14th, 2011, Scheveningen, The Hague (Nederland). Poster (Abstract 1.21).

Moragas AI[1], Salazar O[1], **Jordana-Lluch E[2],** Martró E[2], Molinos S[2], Gimenez M[2], Cospedal R[1], Ausina V[2], Villahermosa ML[1].

1. GENOMICA S.A.U. Coslada. 2. Hospital Universitari Germans Trias i Pujol. Badalona.

Objectives: Accurate and reliable identification of sepsis-causing gram positive bacteria and fungi from positive blood cultures within four working hours using a multiplex-PCR plus microarray based-assay.

Methods: DNA from positive blood cultures was extracted using an automated extractor. Genus-specific primer mixture was designed using conservative regions from bacterial and fungal genomes. The primer design allowed DBNA amplification, producing labelled single stranded DNA product, suitable for microarray hybridization. The probes on the microarray were designed against variable regions flanked by the primers. Specific hibridization were performed in eight well-strip format (CLART-Strip©) containing a microarray at the bottom. Also, *mec*A-specific primers and probes have been concluded in the assay to indicate the detection of methicillin resistance. Automated data analysis was performed by designed specific software.

Results: The assay correctly identifies the most clinically relevant gram positive bacteria (*S. aureus, S. epidermidis, S. haemolyticus, S. pyogenes/dysgalactiae, S. pneumoniae, S. mitis, S. agalactiae, S. sanguinis/parasanguinis, S. milleri, Streptococcus* spp., *E. faecalis, E. faecium, gran positive cocci, L. monocytigenes and Clostridium perfringens*), fungi (*C. albicans, C. glabrata, C. krusei, Candida* spp., generic fungi marker), and the methicillin resistance marker present within Staphylococci, The assay has been clinically validated with 596 positive blood cultures, showing a sensitivity of 97 %, specificity of 100 %, reproducibility of 95.4 % and repeatability of 96.9 %.

Conclusions: The results from the method were available 4 hours after the positive blood culture result, including extraction, amplification and visualization. Up to 96 samples could be processed simultaneously. The assay, therefore, provides rapid, robust and reliable data, which can guide antimicrobial treatment decisions in a timely manner.

Design and optimization of micro array based *in vitro* assay, CLART®*SeptiBac*®, for the rapid identification of gram positive bacteria and fungi present in positive blood cultures.

Ana Isabel Moraga[a], Oscar Salazar[a], Elena Jordana[a], Elisa Martín[a], Sònia Molinos[a] ,Montserrat Giménez[a], Rosario Cospedal[a], Vicente Ausina[b] and M. Luisa Villafermosa[a].

[a]GENOMICA S.A.U. Alcarria 7. Pol. Ind, Coslada, 28823 Coslada, Madrid. osalazar@genomica.com

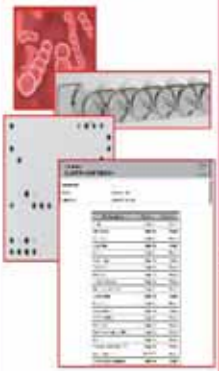
[b]Microbiology Service, University Hospital German Trias i Pujol, 08916 Badalona, Barcelona.

INTRODUCTION

Sepsis is recognized as one of the leading causes of mortality and morbidity in hospitals and is caused mainly by bacteria and fungi. Hospitals usually take a minimum of 24 hours to identify bacterial and fungal pathogens, and the reactions associated with antibiotic resistance. Early detection of pathogens by molecular diagnostic tools can facilitate rapid diagnosis of bacteremia/fungemia, and rapid administration of appropriate antibiotic therapy, reducing inappropriate antibiotic abuse. There are a variety of microbial pathogens associated with sepsis, being necessary the design and optimization of rapid diagnostic tools, for an accurate and reliable microbial detection and identification

OBJECTIVES

Design and development of a multiplex PCR and microarray-based kit for the molecular identification of fungal and gram positive pathogens present in blood cultures.



METHODS

DNA was automatically extracted from blood samples using the equipment NucliSens Easymag (BioMérieux). Multiplex-PCR was optimized using specific primers for each taxa designed from bacterial and fungal house-keeping genes. Amplicons were labelled for further hybridization in the low-density microarray platform (CLART-Strip®).

The probes on the microarray were designed against variable regions flanked by the primers. Specific hybridization were performed in eight well-strip format containing a microarray at the bottom. Also, *mecA*-specific primers and probes have been included in the assay to indicate the detection of methicillin resistance. Automated data analysis was performed by designed specific software. Identification data were compared with original blood culture identification.

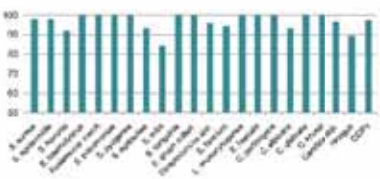
RESULTS

The assay detects and identify 14 grampositive bacterial species belonging to the genera *Streptococcus* (*S. pyogenes, S. dysgalactiae, S. pneumoniae, S. agalactiae, S. mitis, S. sanguinis, S. parasanguinis, Streptococcus milleri* group -*S. anginosus, S. constellatus- and Streptococcus* spp.), *Staphylococcus* (*S. aureus, S. epidermidis, S. haemolyticus, S. hominis* -including the detection of *mecA* in isolates methicillin resistants-), *Clostridium perfringens, Listeria monocytogenes, Enterococcus* (*E. faecium, E. faecalis*), and 3 species of *Candida* genus (*C. albicans, C. glabrata, C. krusei*, including the generic detection of *Candida* spp and universal fungi.

Analytical sensitivity

	100
	1000

Diagnostical sensitivity



After the processing of **596** positive blood cultures, following parameters were obtained:

- Sensitivity = 97%
- Specificity= 100%
- Reproducibility = 95.4%
- Repeatability= 96.9%

CONCLUSIONS:

- The assay shows a high sensitivity, with diagnostical sensitivity from clinical samples over 95%.
- The assay is easily standardized in a clinical laboratory and provide results in 4 hours.
- The trial is multiparametric, allowing simultaneous detection of a large number of pathogens, providing a high robustness, repeatability and reproducibility over 95%.

P1793. Identification and characterisation of bacterial pathogens and fungi causing blood infections (sepsis) by DNA microarrays

22nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), March 31st – April 3rd, 2012, London (United Kingdom). Poster (Abstract P1793).

A.I. Moraga, O. Salazar*, N. Manjon, **E. Jordana,** E. Martró, R. Cospedal, V. Ausina, M. Villahermosa (Coslada – Madrid, Badalona – Barcelona, ES)

Objectives: We have developed a prototype of DNA microarray for accurate and reliable identification of sepsis-causing bacteria and fungi from both samples (positive blood cultures and direct blood) within a working-day using a mutiplex-PCR plus microarray based-assay.

Methods: DNA was automatically extracted from positive blood cultures and blood using different protocols. Species and Genus-specific primer mixture was designed using conservative region from bacterial and fungal genomes. The primer design allowed DNA amplification method producing labeled, single-stranded DNA suitable for microarray hybridization. The probes on the microarray were designed against species-specific or taxa-specific variable regions flanked by the primers. Specific hybridization were performed in eight well-strip format (CLART-Strip©) containing a microarray at the bottom. Also, *mec*A-specific primers and probes have been included in the assay to indicate the detection of antimicrobial resistance. Automated data analysis was performed by designed specific software.

Results: The assay correctly identifies the most clinically relevant species from the following bacteria (*Staphylococus, Streptococcus, Enterococcus, Listeria, Enterobacter, Escherichia, Klebsiella, Salmonella, Citrobacter, Serratia, Proteus, Haemophylus, Bacteroides, Acinetobacter, Pseudomonas* and *Stenotrophomonas*), the methicilin resistance marker present within Staphylococci, and fungi (*Candida albicans albicans. C. glabrata, C. krusei, Candida* spp. and generic fungi marker). The application of this assay for the detection of pathogens directly in blood is being determined by generic PCR assays combined by detection on microarray, being early detection of blood infections crucial in clinical settings. In addition genus and species PCR assays and detection in microarray were made from 700 blood cultures. The results from tested samples were in line with the phenotypic and antimicrobial susceptibility tests, showing values of sensitivity, specificity, reproducibility and repeatability higher than 95 %.

Conclusions: The results from the method were available 5 hours after the positive sample collection. Up to 96 samples could be processed simultaneously. The assay provides rapid and reliable data of accurate identification of microbial pathogens at the early stage of infection, avoiding unnecessary treatment with general antibiotics that promote the appearance of drug resistant bacteria.

Identification and characterization of bacterial pathogens and fungi causing blood infections (sepsis) by DNA microarrays.

Ana Isabel Moragas[a], Nuria Manjón[a], Oscar Salazar[a], Elena Jordana[a], Elisa Martín[a], Montserrat Giménez[b], Rosario Coronel[b], Vicente Asensi[b] and M. Luisa Villahermosa[b].

[a]GENOMICA S.A.U. Alcarria 7, Pol. Ind. Coslada, 28823 Coslada, Madrid. oxalazar@genomica.com

[b]Microbiology Service, University Hospital Germans Trias i Pujol, 08916 Badalona, Barcelona.

INTRODUCTION

Sepsis is recognized as one of the leading causes of mortality and morbidity in hospitals and is caused mainly by bacteria and fungi. Hospitals usually take a minimum of 24 hours to identify bacterial and fungal pathogens, and the reactions associated with antibiotic resistance. Early detection of pathogens by molecular diagnostic tools can facilitate rapid diagnosis of bacteremia/fungaemia, and rapid administration of appropriate antibiotic therapy, reducing inappropriate antibiotic abuse. There are a variety of microbial pathogens associated with sepsis, being necessary the design and optimization of rapid diagnostic tools, for an accurate and reliable microbial detection and identification.

OBJECTIVES

•Design and development of a multiplex PCR and microarray-based kit for the molecular identification of bacterial and fungal pathogens present in positive blood cultures.

•Optimization of DNA extraction method from blood samples for testing the above described assay in order to define the analytical sensitivity (cfu/ml) in simulated blood infections using sterile blood samples spiked with defined loads of microorganisms as inoculum.

METHODS

•**Blood cultures**: DNA was automatically extracted from blood samples using the equipment NucliSens Easymag (BioMérieux).

•**Simulated blood infections**: sterile blood samples were spiked with known concentrations of fungal and bacterial pathogens (Macfarland standard), and DNA were pre-extracted using a modificated protocol with MAYAtec extraction kit (Mistrymé) followed by automatic extraction with NucliSens Easymag equipment.

Multiplex-PCR was optimized using specific primers for each taxa designed from bacterial and fungal house-keeping genes. Amplicons were labeled for further hybridization in the low-density microarray platform (CLART-Skip®).

The probes on the microarray were designed against variable regions flanked by the primers. Specific hybridization were performed in eight well-strip format containing a microarray at the bottom. Also, *mecA*-specific primers and probes have been included in the assay to indicate the detection of methicillin resistance. Automated data analysis was performed by designed specific software.

RESULTS

•**Blood cultures**: the assay detects and correctly identifies 14 gram positive bacterial species, 15 gram negative bacterial species and 3 species of *Candida* genus including the generic detection of *Candida* spp. and universal fungi. The analytical and diagnostical parameters obtained for each taxa are summarized in **Table 1**.

•**Simulated blood infections**: results of analytical sensitivity are sumarized in **Table 2**, ranging between 14 and 63 cfu/ml.

Table 2: ANALYTICAL SENSITIVITY FROM SIMULATED BLOOD INFECTIONS

Microorganisms	Sensitivity (cfu/ml)
Staphylococcus aureus	16
Staphylococcus epidermidis	63
Staphylococcus spp.	63
Streptococcus pyogenes	63
Streptococcus dysgalactiae	16
Streptococcus pneumoniae	63
Streptococcus spp.	63
Streptococcus agalactiae	16
Streptococcus mitis/ group	16
Enterococcus faecalis	16
Clostridium perfringens	63
Enterobacter aerogenes	16
Enterobacter cloacae	63
Serratia marcescens	16
Proteus mirabilis	16
Escherichia coli	16
Stenotrophomonas maltophilia	16
Candida albicans	63
Candida spp.	63
Fungi	63

[illegible] 100% (3 samples tested). Haemophilus influenzae 100% (3 samples tested). Stenotrophomonas maltophilia 100% (2 samples tested).

CONCLUSIONS:

•The assay shows high values of sensitivity and specificity for the detection and identification of microbial pathogens from positive blood cultures, being easily standardizable in routine laboratory, with a total processing time of 4 hours.

•Preliminary and promising data using simulated blood infections provide high analytical sensitivity, allowing further optimization of the assay using real septic blood samples from patients for the early detection of sepsis causing pathogens.

-245-

740. Diseño y optimización de un sistema de detección molecular por microarrays para la rápida identificación de bacterias Gram-negativas en hemocultivos positivos

XVI Nacional de la Sociedad de Enfermedades Infecciosas y Microbiologia Clínica (SEIMC), 9-11 Mayo, 2012, Bilbao. Poster (Abstract N.º 740).

O. Salazar Torres[1], N. Manjón Vega[1], A.I. Moraga Quintanilla[1], **E. Jordana Lluch[2],** E. Martro Catalá[2], S. Molinos Abós[2], M. Giménez Pérez[2], R. Cospedal García[1], V. Ausina Ruiz[2] y M.L. Villahermosa Jaén[1]

1. Genomica SAU. Coslada. Madrid. 2. Hospital Universitari Germans Trias i Pujol. Badalona. Barcelona.

Objetivos: Diseño y optimización de un método de identificación rápida a nivel de género y especie de bacterias Gram-negativas a partir de hemocultivos positivos.

Métodos: el ADN bacteriano fue extraído de manera automática a partir de hemocultivo positivo usando el extractor automático Nucli-SENS. easyMAG. de bioMérieux. Se ha optimizado una multiplex-PCR a partir de cebadores específicos para cada taxón diseñados en regiones constitutivas del genoma bacteriano. Los productos amplificados fueron marcados para su posterior hibridación en una plataforma de microarrays de baja densidad (CLART-Strip.). Las sondas específicas para cada taxón, e inmovilizadas en el microarray fueron diseñadas a partir de regiones flanqueantes a la zona de hibridación de los cebadores. Las hibridaciones específicas fueron realizadas en formato de tiras de 8 pocillos con el microarray impreso en el fondo del pocillo. Se ha incluido el análisis automático de datos mediante el diseño de una aplicación informática específica.

Resultados: El ensayo detecta correctamente 15 especies de bacterias Gram-negativas presentes en hemocultivos positivos: *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter aerogenes, Enterobacter cloacae, Salmonella enterica, Citrobacter freundii, Serratia marcescens, Proteus vulgaris, Proteus mirabilis, Haemophilus influenzae, Acinetobacter baumannii, Bacteroides fragilis, Pseudomonas aeruginosa y Stenotrophomonas maltophilia.* El ensayo ha proporcionado una sensibilidad y especificidad analítica cercana al 100 % a una concentración de 103 copias por reacción para plásmidos recombinantes control desarrollados que contienen las dianas de amplificación de las especies objeto de detección del kit. La sensibilidad y especificidad diagnóstica en hemocultivos en la mayoría de los microorganismos detectados es del 95 %.

Conclusiones: Los resultados de identificación se obtuvieron 4 horas después de la detección del hemocultivo como positivo. El ensayo permite el procesamiento de hasta 96 arrays en una misma tanda, siento la unidad mínima de ensayo la tira de 8 arrays, proporcionando una identificación rápida y fiable, permitiendo una aplicación más dirigida de terapia antimicrobiana antes del aislamiento del microorganismo.

Diseño y optimización de un sistema de detección molecular por microarrays para la rápida identificación de bacterias gram-negativas en hemocultivos positivos.

Oscar Salazar[a], Nuria Mauleón[a], Ana Isabel Moreno[a], Elena Jordana[a], Elisa Martró[b], Sonia Molinos[b] Montserrat Gimenez[b], Rosario Cespedal[a], Vicente Ausina[b] and M. Luisa Villanueva[a].

[a] GENOMICA S.A.U. Alcarria 7. Pol. Ind. Coslada. 28822 Coslada, Madrid. mmanjon@genomica.com

[b] Servicio de Microbiología. Hospital Universitario Germans Trias i Pujol. 08916 Badalona, Barcelona.

INTRODUCCION

La sepsis es reconocida como una de las principales causas de mortalidad y morbilidad en los hospitales y es causada principalmente por bacterias y hongos. Los hospitales suelen tardar un mínimo de 24 horas para identificar bacterias y hongos patógenos y las reacciones asociadas con resistencia a los antibióticos. La detección temprana de patógenos mediante las herramientas de diagnóstico molecular puede facilitar el diagnóstico rápido para establecer una terapia antibiótica apropiada y de este modo la reducción del abuso inadecuado de antibióticos. Hay una variedad de patógenos microbianos asociados con la sepsis, siendo necesario el diseño y optimización de herramientas de diagnóstico rápido, para una detección microbiana exacta y fiable y de identificación.

OBJETIVOS

•Diseño y optimización de un método de identificación rápida a nivel de género y especie de bacterias Gram-negativas a partir de hemocultivos.

METODOS

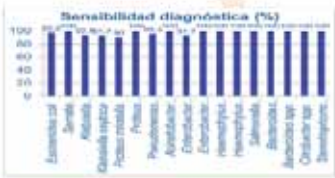
El ADN bacteriano fue extraído de manera automática a partir de hemocultivo usando el extractor automático Nucli*SENS*® *easyMAG*® de Biomérieux. Se ha optimizado una múltiplex-PCR a partir de cebadores específicos para cada taxón diseñados en regiones constitutivas del genoma bacteriano. Los productos amplificados fueron marcados para su posterior hibridación en una plataforma de microarrays de baja densidad (CLART-Strip®). Las sondas específicas para cada taxón, o inmovilizadas en el microarray fueron diseñadas a partir de regiones flanqueantes a la zona de hibridación de los cebadores.

Las hibridaciones específicas fueron realizadas en formato de tiras de 8 pocillos con el microarray impreso en el fondo del pocillo. Se ha incluido el análisis automático de datos mediante el diseño de una aplicación informática específica.

RESULTADOS

El ensayo detecta correctamente 22 especies de bacterias Gram-negativas presentes en hemocultivos. *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca, Enterobacter aerogenes, Enterobacter cloacae, Salmonella enterica, Citrobacter freundii, Citrobacter spp. (C.freundii, C. koseri), Serratia marcescens/plymuthica, Proteus vulgaris/penneri, Proteus mirabilis, Haemophilus influenzae, Haemophylus spp.(H. influenzae, H.parainfluenzae) Acinetobacter baumanii, Bacteroides fragilis, Bacteroides spp. (B. fragilis, B. faecis), Pseudomonas spp. (P. aeruginosa, P. putida, P. stuartii)y Stenotrophomonas maltophilia.* El ensayo ha proporcionado una sensibilidad y especificidad analítica del 100% a una concentración de $10\text{-}10^3$ copias por reacción para plásmidos recombinantes control desarrollados, que contienen las dianas de amplificación de las especies objeto de detección del kit. La especificidad diagnóstica en hemocultivos es mayor del 99% y la sensibilidad diagnóstica mayor del 90%.

Sensibilidad analítica	Copias por reacción
Enterobacter cloacae	10
Serratia marcescens	
Proteus mirabilis	
Proteus vulgaris	
Pseudomonas aeruginosa	
Haemophylus influenzae	100
Enterobacter aerogenes	
Citrobacter freundii	
Klebsiella pneumoniae	
Salmonella enterica	
Escherichia coli	
Acinetobacter baumanii	
Bacteroides fragilis	
Stenotrophomonas maltophilia	
Klebsiella oxytoca	1000



Análisis de 299 muestras clínicas:
- ✓ Sensibilidad >90%
- ✓ Especificidad 100%
- ✓ Reproducibilidad > 94,6%
- ✓ Rapidez/tiempo < 24h

CONCLUSIONES.

Los resultados de identificación se obtuvieron en 4 horas después de la detección del hemocultivo como positivo. El ensayo permite el procesamiento de hasta 96 arrays en una misma tanda, siendo la unidad mínima de ensayo la tira de 8 arrays, proporcionando una identificación rápida y fiable y permitiendo una aplicación más dirigida de terapia antimicrobiana antes del aislamiento del microorganismo.

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736. Diseño y optimización de un sistema de detección molecular por microarrays para la rápida identificación de bacterias Gram-positivas, Gram-negativas y hongos en muestras de sangre

Congreso Nacional de la Sociedad de Enfermedades Infecciosas y Microbiologia Clínica SEIMC), 9-11 Mayo, 2012, Bilbao. Poster (Abstract N.º 736).

N. Manjón Vega[1], A.I. Moraga[1], O. Salazar[1], **E. Jordana[2],** E. Martró[2], M. Giménez[2], R. Cospedal[1], V. Ausina[2] y M.L. Villahermosa[1]

1. Genomica SAU. Coslada. Madrid 2. Hospital Germans Trias y Pujol, Badalona. Barcelona

Objetivos: Diseño y optimización de un método de identificación rápida a nivel de género y especie de bacterias Gram-positivas, Gram-negativas y hongos a partir de muestras de sangre.

Métodos: El DNA bacteriano fue extraído de manera automática tras un pre-tratamiento previo, a partir de sangre, usando el extractor automático NucliSENS. easyMAG. de Biomérieux. Se ha partido de muestras de sangre de pacientes sanos contaminadas, según la escala de McFarland 0,5 ($1,5 \times 10^8$ UFC/mL), con diferentes diluciones de cada uno de los microorganismo (500 - 16 UFC/mL). Se ha optimizado una multiplex-PCR a partir de cebadores específicos para cada taxón dise.ados en regiones constitutivas del genoma bacteriano y fúngico. Los productos amplificados fueron marcados para su posterior hibridación en una plataforma de microarrays de baja densidad (CLART-Strip.). Las sondas específicas para cada taxón, e inmovilizadas en el microarray fueron diseñadas a partir de regiones flanqueantes a la zona de hibridación de los cebadores. Las hibridaciones específicas fueron realizadas en formato de tiras de 8 pocillos con el microarray impreso en el fondo del pocillo. Se han incluido cebadores y sondas específicas para la detección específica del gen *mec*A, responsable de la resistencia a meticilina en *Staphylococcus aureus.* Se ha incluido el análisis automático de datos mediante el diseño de una aplicación informática específica.

Resultados: El ensayo detecta correctamente 10 especies de bacterias Gram-positivas pertenecientes a los géneros *Streptococcus* (*S. pyogenes, S. dysgalactiae, S. pneumoniae, S. agalactiae, S. mitis, Streptococcus* del grupo *milleri* (*S. anginosus, S. constellatus*), *Staphylococcus* (*S. aureus, S. epidermidis, S. haemolyticus*), incluyendo la detección de *mec*A, *Clostridium perfringens, Enterococcus faecalis,* 8 especies de bacterias Gram-negativas *Enterobacter aerogenes, Enterobacter cloacae, Serratia marcescens, Proteus mirabilis, Bacteroides fragilis* y *Stenotrophomonas maltophilia* y *Candida albicans,* incluyendo la detección genérica de *Candida* spp, y hongos en general. El ensayo ha proporcionado una sensibilidad y especificidad analítica de un 100 % y una sensibilidad y especificidad diagnóstica superior 63 UFC/mL.

Conclusiones: Los resultados de identificación se obtienen tras 8 horas de la recogida de la muestra. El ensayo permite el procesamiento de hasta 96 arrays en una misma tanda, siento la unidad mínima de ensayo la tira de 8 arrays, y proporcionando una identificación rápida y fiable, la cual permitirá la aplicación de un tratamiento más dirigido a nivel de terapia antimicrobiana en los estadios tempranos de la infección.

Diseño y optimización de un sistema de detección molecular por microarrays para la rápida identificación de bacterias gram-positivas, gram-negativas y hongos en muestras de sangre.

Nuria Mencia[1], Ana Isabel Moragó[1], Oscar Salvacer[1], Elena Jordana[2], Elisa Martín[2], Montserrat Giménez[2], Rosario Coupeau[2], Vicente Auzina[2] and M. Luisa Villalmanzo[2].

[1] GENOMICA S.A.U. Alcarria 7, Pol. Ind. Coslada, 28823 Coslada, Madrid. nmartyn@genomica.com

[2] Servicio de Microbiología. Hospital Universitario Germans Trias i Pujol, 08916 Badalona, Barcelona

INTRODUCCION

La sepsis es una de las principales causas de morbilidad en pacientes hospitalizados, con una mortalidad cercana al 50% en casos de shock séptico. Con las técnicas de detección basadas en hemocultivos se tarda entre 24 y 72 horas en identificar el patógeno causante de la infección, por ello se administran antibióticos de amplio espectro como terapia inicial antes de identificar a dicho microorganismo. En cambio, las técnicas moleculares proporcionan una identificación rápida del patógeno, permitiendo establecer una terapia antimicrobiana apropiada desde el inicio. Son muchos los patógenos microbianos asociados con la sepsis y es necesario diseñar herramientas de diagnóstico rápido, para una detección microbiana exacta y fiable.

OBJETIVOS

•Optimización de un método de extracción de DNA a partir de muestras de sangre de pacientes sanos inoculadas con diferentes diluciones del microorganismo, con el fin de definir su sensibilidad diagnóstica (ufc/ml).

•Desarrollo de un sistema de identificación rápida a nivel de género y especie de bacterias Gram-positivas, Gram-negativas y hongos a partir de muestras de sangre.

METODOS

El ADN bacteriano fue extraído de manera automática tras un pre-tratamiento previo, a partir de sangre, usando el extractor automático NucliSENS® easyMAG® de Biomerieux. Se ha partido de muestras de sangre de pacientes sanos contaminadas, según la escala de McFarland 0.5 (1.5 x 10^8 ufc/ml), con diferentes diluciones de cada uno de los microorganismos (500-16 ufc/ml). Se ha optimizado una multiplex-PCR a partir de cebadores específicos para cada taxón diseñados en regiones constitutivas del genoma bacteriano y fúngico. Los productos amplificados fueron marcados para su posterior hibridación en una plataforma de microarrays de baja densidad (CLART-Strip®). Las sondas específicas para cada taxón, e inmovilizadas en el microarray, fueron diseñadas a partir de regiones flanqueantes a la zona de hibridación de los cebadores.

Las hibridaciones específicas fueron realizadas en formato de tiras de 8 pocillos con el microarray impreso en el fondo del pocillo. Se han incluido cebadores y sondas específicas para la detección específica del gen mecA, responsable de la resistencia a meticilina en *Staphylococcus aureus*. Se ha incluido el análisis automático de datos mediante el diseño de una aplicación informática específica.

RESULTADOS

•El ensayo detecta correctamente 8 especies de bacterias Gram-positivas pertenecientes a los géneros *Streptococcus (S. pyogenes, S. dysgalactiae, S. pneumoniae, S. agalactiae, Streptococcus del grupo milleri (S. anginosus, S. constellatus)), Staphylococcus (S. aureus, S. epidermidis)*, incluyendo la detección de mecA, *Clostridium perfringens, Enterococcus faecalis, 7* especies de bacterias Gram-negativas *Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae, Serratia marcescens, Proteus mirabilis, Bacteroides fragilis y Stenotrophomonas maltophilia* y *Candida albicans*, incluyendo la detección genérica de *Candida spp*, y hongos en general.

•El ensayo ha proporcionado una sensibilidad diagnóstica del 100% superior a 63 ufc/ml con una especificidad del100%.

Sensibilidad analítica	
Microorganismos	Sensibilidad (ufc/ml)

CONCLUSIONES:

Los resultados de identificación se obtienen tras 8 horas de la recogida de la muestra. El ensayo permite el procesamiento de hasta 96 arrays en una misma tanda, siendo la unidad mínima de ensayo la tira de 8 arrays, y proporcionando una identificación rápida y fiable, la cual permitirá la aplicación de un tratamiento más dirigido a nivel de terapia antimicrobiana en los estadios tempranos de la infección.

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S'ha utilitzat la tipografia Cardo, elaborada per David Perry, i de lliure accés i distribució.