Curr Treat Options Infect Dis (2017) 9:1–10
DOI 10.1007/s40506-017-0104-1

Update in Bloodstream Infection Diagnosis Using New Methods in Microbiology

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Published online: 27 January 2017
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This article is part of the Topical Collection on *New Technologies and Advances in Infection Prevention*

Keywords Bloodstream infection · Molecular diagnosis · Stewardship · Molecular panels · Polymerization chain reaction

Opinion statement

Bloodstream infections remain an important cause of morbidity/mortality worldwide. The diagnosis of these infections is time-consuming, even with the use of automated blood culture systems. Several systems based on molecular biology and, more recently, proteomics have been developed to allow rapid and accurate diagnosis of bloodstream infections. Here, we describe some recently technologies and commercial systems available to detect and to identify microorganisms and bacterial antimicrobial resistance-coding genes from positive blood culture bottles and whole-blood samples. Evaluation of clinical outcomes in multicenter studies and clinical trials with these new tests is warranted in conjunction with antimicrobial stewardship use and programs for interpretation of results to be provided to physicians.

Introduction

Despite medical advances in recent decades, bloodstream infections (BSIs) are still important causes of morbidity and mortality worldwide. Recent studies have reported rates between 80 and 189 per 100,000 per year with higher rates reported in more recent years [1]. These infections are associated with high morbidity and mortality and increase overall health care costs, and the early diagnosis of BSIs is essential for the institution of appropriate therapy [2].

Historically, blood culture is the gold standard method for diagnosing BSIs. However, the delay between blood culture sampling and final identification and susceptibility testing of the organism responsible for the bacteremia by the current classical procedure is time-

Table 1. Current molecular tests for diagnosis of bloodstream infections

Assay manufacturer	Technology	Number of pathogens detected	Resistance markers	Turnaround time[a]
Positive blood culture assays				
Verigene Gram-negative/ positive Blood Culture (Nanosphere Inc., USA)	Multiplex real-time PCR	9 GP and 13 GN	mecA, vanA/B, CTX-M, IMP, KPC, NDM, OXA, and VIM	2.5 h
FilmArray Blood Culture Identification (BioFire Diagnostics, USA)	Multiplex real-time PCR + hybridization on Microarray	8 GP, 11 GN, and 5 yeasts	mecA, vanA, vanB, and KPC	1 h
PNA-FISH (AdvanDx, USA)	Fluorescence-based hybridization with PNA probes	4 GP, 3 GN, and 5 yeasts	None	1.5–3 h
QuickFISH (AdvanDx, USA)	Fluorescence-based hybridization	4 GP and 3 GN	None	20 min
Plex-ID (Abbott, USA)	Multiplex PCR + electrospray ionization mass spectrometry	Multiple pathogens	mecA and van	<3 h
Hyplex Blood Screen (BAG, Germany)	Multiplex PCR + hybridization	6 GP and 5 GN	mecA and van	3 h
Prove-it Sepsis (Mobidiag, Finland)	Multiplex real-time PCR + hybridization on microarray	60 bacteria and 13 fungi	mecA	3 h
Black-Light Sepsis Kit (BlackBio, Spain)	Broad-range PCR and pyrosequencing	Multiple pathogens	None	4 h
MALDI-TOF (Bruker Daltonics, Germany, or bioMérieux, France)	Mass spectral signal recognition, laser desorption ionization, mass spectrometry	Multiple pathogens	None[b]	<1 h
Whole-blood assays				
SeptiTest (Molzyn, Germany)	Broad-range PCR and sequencing	>300 pathogens	None	8–12 h
Light Cycler SeptiFast (Roche, Germany)	Multiplex real-time PCR	8 GP, 10 GN, 5 yeasts, and *Aspergillus fumigatus*	mecA	6 h
T2 *Candida* panel (T2 Biosystems)[c]	Magnetic resonance + nanotechnology	5 yeasts	None	3–5 h
Vyoo (SIRS lab, Jena, Germany)	Multiplex PCR + gel electrophoresis	34 bacteria and 6 fungi	mecA, vanA/B/C, and SHV	7 h
IRIDICA (Abbott, USA)	Multiplex PCR + electrospray ionization mass spectrometry	Multiple pathogens	mecA, vanA/B/C, and KPC	8–12 h
VAPChip (Eppendorf, Belgium)	Multiplex PCR + hybridization on microarray	2 GP and 11 GN	TEM, CTX, SHV, mecA, and carbapenemases	5–8 h
Magicplex (Seegene, Korea)	Multiplex real-time PCR	73 GP, 11 GN, and 6 fungi	mecA and vanA/B	3–4 h

GP Gram positive, *GN* Gram negative
[a]After blood culture turns positive
[b]In development
[c]Research use only

consuming, even with the use of automated systems [3]. This methodology implies a delay of up to 48–72 h in the result and has several limitations regarding sensitivity, especially in the case of previous antimicrobial therapy, and fastidious, slow-growing, or uncultivable pathogens, often leading to a low diagnostic yield, despite the many improvements that have been proposed to speed up and standardize these processes [4–7].

This short review aims to sketch some recent developments in laboratory-based clinical bacteriology and to provide an overview of emerging approaches for diagnosis of BSIs.

Molecular approaches

Molecular biology for diagnosis of BSIs is based on amplification of nucleic acids, species-specific hybridization, micro-array technology, and gene sequencing (e.g., 16S ribosomal RNA gene), and several systems based on these techniques have been developed. They allow rapid and sensitive detection of microorganisms, including bacteria, as well as viruses, parasites, and fungi. Most of them give the result from the agent grown in the blood culture, while others are capable to identify bacteria present directly from not-incubated peripheral blood [8–10, 11••].

The polymerization chain reaction (PCR) offers a rapid and reliable alternative to conventional culture, reducing the time to detection and increasing the sensitivity in the identification of certain microorganisms and antimicrobial resistance-coding genes.

From the routine use of PCR, other methods and applications could be developed. One is the multiplex PCR that constitutes the amplification of more than one target simultaneously by adding different sets of specific primers for each target in the same test.

The quantitative real-time PCR (qPCR) is a refinement of the original technique of PCR as it combines amplification and quantification of a target DNA sequence by means of fluorescence detection using specific probes tagged with fluorescence or based on the determination of denaturation temperature of a sequence of double-stranded DNA (the melting temperature) labeled with fluorescent intercalating substance. It is considered a homogeneous method of DNA amplification, in which amplification and detection are carried out in the same reaction tube and can eliminate post-PCR processing, reducing the handling of the amplification products and the risk of cross-contamination.

Currently, some commercial platforms based on molecular approaches are available. They constitute accurate and sensitive panels with specific and low manipulated assays short-incubated on specifically easy-to-use designed instruments. Rapid results, in a 1 to 2.5-h time frame, are available when using these technologies. But as limitations, they are high cost and closed trading platforms that provide identification just for some relevant microorganisms and resistance genes.

Two platforms are approved by the FDA for testing positive blood culture bottles: The FilmArray Blood Culture Identification Panel (BioFire Diagnostics, USA) and the Verigene Gram-Positive Blood Culture Test and Gram-Negative Blood Culture Test (Nanosphere Inc., USA). The FilmArray constitutes a multiplex PCR system that integrates sample preparation, amplification, detection, and analysis. It comprehends tests for a variety of pathogens that cause BSIs, as

well as antimicrobial resistance genes in about an hour [12].

The Verigene platform is a patented technology workstation that uses gold nanoparticles to detect pathogens and drug resistance markers. It combines automated nucleic acid extraction, purification, amplification (if required), and hybridization divided into two modules: one for Gram positive and other for Gram negative [13].

Other platforms can be applied to positive blood culture bottles, but they still remain under clinical validation and use approval. They are summarized in Table 1.

Molecular tests performed from positive culture bottles represent sensitive tools but are inefficient for some patients in which the culture remains negative because of infections caused by fastidious or slow-growing agents or those who need more rapid results. For these infections, tests directly from the peripheral blood sample would be most applicable. Although these assays provide results more rapidly, the low bacterial density in blood during bacteremia (1–10 CFU/mL) often results in low sensitivity

Several assays targeting specific pathogens and resistance genes have been developed for clinical samples without previous culture such as EDTA blood. The SepsiTest (Molzym, Germany) and the Light Cycler SeptiFast (Roche, Germany) are some available panels based on broad-range PCR.

The SeptiTest platform is based on four steps: DNA extraction, Universal PCR amplification targeting the 16S rRNA (for bacteria) and the 18S ribosomal DNA (rDNA) (for fungi), Sanger sequencing, and online identification. The test is able to identify species from more than 200 genera of bacteria and 65 genera of fungi. However, this is an expensive platform that requires careful handling but gives a rapid result, in about 5 to 12 h. The analytical sensitivity ranges from 10 to 80 CFU/mL, depending on the target species and false-positive results, are reported [14].

The SeptiFast system involves three distinct processes: specimen preparation by mechanical lysis and purification of DNA, real-time PCR amplification in three parallel reactions targeting the internal transcribed spacer region between the 16S and 23S rDNAs of Gram-positive and Gram-negative bacteria and 18S rDNA sequence of fungi, and detection using fluorescence-labeled probes specific to the target DNA. Previous studies using SeptiFast in different kinds of patient populations report a concordance of 70 to 88% within the blood culture results [14–16].

A novel qPCR assay, the Xpert MRSA/SA BC (Cepheid, USA), which detects methicillin-resistant or methicillin-susceptible *Staphylococcus aureus* from positive blood bottles, was also described applied directly to whole-blood samples to diagnose catheter-related bacteremia with 87.5% of sensitivity and 92.1% of specificity [17].

Beyond the approved panels, several in-house assays have been frequently reported to detect and identify microorganisms and resistance genes both from blood culture bottles and whole-blood samples. These assays often use open qPCR instruments such as 7500 Applied Biosystems and 6000 Rotor Gene (Qiagen, USA) that enable adjustment in the test according to the patient population or local epidemiology, as well as lower cost. Our laboratory group has explored this tool by developing a protocol for rapid diagnosis of BSIs using in-house qPCR with Gram-specific probes, followed by TaqMan-based reactions for the agent identification and resistance genes. Good results were

obtained from blood culture bottles, while minor sensibility was observed among peripheral blood samples [10, 11••].

In this context, the qPCR applied to clinical sample testing presents another challenge: the fully DNA extraction without contamination. Some studies have been reporting solutions to this problem by using more sensitive methods of DNA extraction from clinical samples, even introducing additional PCR steps [18]. The introduction of automated extraction devices like the easyMAG (Biomerieux, France) and M2000 (Abbot, USA) and apparatus in which the DNA extraction and qPCR are coupled in a single step (BD MAX, Becton, Dickinson) represents alternatives to these difficulties. They enable good sensitivity and specificity in the detection of genes of interest and could favor the introduction of molecular diagnosis in clinical microbiology laboratories [19, 20].

Broad-range assays require an initial DNA amplification followed by further steps like DNA sequencing or hybridization and these technologies are not avaiable in all clinical laboratories. So, tests based in fluorescence in sito hybridization (FISH) appears as an alternative. Peptide nucleic acid fluorescent probes (PNAFISH; AdvanDx, USA) are synthetic oligomers that mark the microbial DNA or RNA present in the sample, detecting these microorganisms without the need for previous steps, and are thus less likely to be affected by contamination. However, the benefit of these rapid assays remains unclear and needs to be better evaluated from an antibiotic stewardship program [21].

Mass spectroscopy

Apart from genomics, the proteomics also gained ground in clinical laboratories and the mass spectrometry (MS) stands out. It is a novel technique in the detection of markers for diseases in which the diagnosis is performed by invasive methods and requires speed results. In microbiology, wherein the identification of isolates by biochemical tests depends on metabolic microorganism processes, the MS appears as a rapid and reliable tool.

Since its appearance in 1902, the MS has undergone several modifications and improvements and was first used in microbiological identification in 1975. Techniques such as matrix-assisted laser desorption ionization time of flight (MALDI-TOF) appeared in 1980 and allowed the precise identification of high-weight molecules. However, only in the seven past years that the technique became available for use in microbiology longer to be the domain only of MS experts [22•].

The types of spectrometers MALDI-TOF are the most common for identification and classification of microorganisms. The abbreviation refers to MALDI ionization process by laser desorption matrix assisted; that is, the matrix is an organic acid which provides a proton for the sample ionization process when excited by base. Already, the acronym TOF features the flight time of the ionized sample into a vacuum tube until it reaches the detector.

MALDI-TOF utilizes MS to rapidly identify organisms following isolation from clinical specimens. MALDI-TOF also accurately and promptly identifies most bacterial and yeast species and resistance profiles directly from blood culture bottles and represents an attractive alternative to more time-consuming conventional testing methods [22•, 23].

For the identification of these molecules, databases assembled by branch companies of MS are used and remain to have constant updates containing bacteria mass spectra. The principal MS systems are BIOTYPER (Bruker Daltonics, Bremen, Germany) and the VITEK MS (bioMérieux, Marcy l'Etoile, France). VITEK MS is a device installed on the floor and offers two database systems to query: Myla (IVD) and Saramis (RUO), but these were combined recently in VITEK MS Plus.

Due the currently use of MS, new information storage and constant update of database, further studies are carried out and its use in microbiology is not just for bacterial identification, but also for determination of enzymes that promote antimicrobial resistance in these organisms. Recent studies further demonstrate the applicability of this technique when performed directly from the blood culture vials to detect species of microorganisms and antimicrobial resistance mechanisms [23, 24].

Emerging technologies

The development of new diagnostic systems for diagnosis of BSIs remains ongoing. Emerging technologies, i.e., PCR-electrospray ionization mass spectrometry (PCR-ESI MS) and next-generation sequencing (NGS), appear to be some technologies that might solve the failures of the currently available DNA-based molecular assays.

Like MALDI-TOF MS, the PCR ESI-MS is a form of soft ionization which lends itself to analysis of larger macromolecules, including proteins and nucleic acids. It measures the mass/charge ratio of generated PCR amplicons originating from several conserved and species-specific regions of microorganisms genomes. The obtained spectra are compared to those present in a specific database.

The first PCR/ESI-MS system was developed by Abbott, the PLEX-ID, but showed suboptimal performance [25]. Subsequently, the system was redesigned as IRIDICA BAC BSI and identifies +750 bacterial species, *Candida*, and resistance markers from 5 mL of EDTA blood in a 6-h turnaround [14, 26].

The T2 magnetic resonance (T2 Biosystems, MA, USA) is other new technology capable of detecting yeast species directly from whole-blood samples. The system is based on a miniaturized, magnetic resonance that measures how water molecules react in the presence of magnetic fields. The technology qualitatively detects five species of *Candida* and promises to have a major clinical impact resulting from the diagnosis of previously unrecognized deep-seated candidiasis as well as from the real-time detection of candidemia [27].

Whole-genome sequencing and metagenomic analysis is quickly taking place of methods for typing microorganisms, but only sporadic studies report the use of NGS in clinical bacteriology testing. NGS instruments consist of open platforms (e.g., MiSeq System (Illumina Inc.), the Ion Torrent Personal Genome Machine (Life Technologies), the Ion Proton System (Life Technologies), and others) that provide detection and

quantification from any DNA sequence information within patient specimens. It is potentially a high-sensitivity and high-specificity method for diagnosis, but there are as yet no commercially available systems for sequencing in clinical bacteriology laboratories [22•, 28].

The clinical impact

These rapid molecular tests are expected to impact the mortality rate of bloodstream infections (BSIs). However, few studies were designed to evaluate the clinical outcome. In a systematic review published in 2015, which considered mortality as the primary outcome, the authors verified that only studies comparing rapid test in conjunction with antimicrobial stewardship programs showed a mortality benefit [2].

Integration of MALDI-TOF into clinical microbiology workflow decreases time to organism identification by 1.2–1.5 days compared to conventional methods. However, there are limited data evaluating the impact of MALDI-TOF implementation on patient outcomes. An adequate therapy is reported in 35% of cases of bacteremia when using results of MALDI-TOF, despite the previous Gram staining [29]. Other study also observed a significant impact of the result of the identification by MALDI-TOF MS directly from the blood culture bottle in modifying the antimicrobial therapy of patients with ICS [30].

Vlek and colleagues using MALDI-TOF MS for identification of microorganisms from blood culture bottles showed an 11.3% increase in the proportion of patients receiving appropriate antibiotic treatment 24 h after blood culture positivity [29]. Also, Huang and colleagues evaluated the rapid microorganism identification associated with a pre- and post-intervention stewardship team; the mortality, length of intensive care unit stay, and recurrent bacteremia were lower within the intervention group [31].

The Verigene Gram-positive blood culture assay has a potential use for antibiotic stewardship in patients with Gram-positive bacteremia including stopping or preventing unnecessary vancomycin or starting a targeted therapy [32]. Also, Bork and colleagues in a retrospective designed study demonstrated the combination of the Verigene Gram-negative blood culture assay with antimicrobial stewardship intervention in Gram-negative bacteremia [33]. A clinical trial was designed utilizing the Verigene Gram-positive and Gram-negative blood culture tests and revealed an earlier time of appropriate antimicrobial agent initiation and a lower 30-day mortality rate in the intervention period [34].

The film array for blood culture pathogen identification was also evaluated comparing strategies to communicate rapid test results to clinicians with no statistical difference in clinical outcomes [35].

A European multicenter study evaluated the PCR/ESI-MS: Plex ID from a single 5-mL EDTA blood sample with results within 6 h. The clinical value was assessed by a panel of three independent infectious disease microbiologists and intensive care specialists. A change in antimicrobial prescription was recommended in 41% of cases with a percentage increase to 57 when the molecular test was positive [36••].

In-house real-time PCR protocols can be utilized, particularly for screening of antimicrobial resistance-coding genes after identification of microorganisms

from blood bottles. In a retrospective study in children with cancer, our group showed potential benefits in clinical outcomes utilizing a multiplex protocol [37•].

Conclusion

New methodologies and platforms are available for rapid and accurate diagnosis of BSIs. Most of them were analytically validated, including in-house protocols; however, they still require more clinical data to be applied in clinical microbiology routine with a positive cost/benefit. Multicenter studies and clinical trials are warranted including stewardship programs for interpretation of these new test results to be provided to physicians for decision making.

Compliance with Ethical Standards

Conflict of Interest
Milene Quiles declares that she has no conflicts of interest.
Bruno Boettger declares that he has no conflicts of interest.
Antonio Carlos Campos Pignatari declares that he has no conflicts of interest.

Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

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•• Of major importance

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