CRITICAL CARE

REVIEW

Bench-to-bedside review: Rapid molecular diagnostics for bloodstream infection - a new frontier?

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Abstract

Among critically ill patients, the diagnosis of bloodstream infection poses a major challenge. Current standard bacterial identification based on blood culture platforms is intrinsically time-consuming and slow. The continuous evolvement of molecular techniques has the potential of providing a faster, more sensitive and direct identification of causative pathogens without prior need for cultivation. This may ultimately impact clinical decision-making and antimicrobial treatment. This review summarises the currently available technologies, their strengths and limitations and the obstacles that have to be overcome in order to develop a satisfactory bedside point-of-care diagnostic tool for detection of bloodstream infection.

Introduction

Bloodstream infections (BSIs) are associated with adverse clinical and health-economic outcomes [1,2]. Thirty to forty percent of all cases of severe sepsis and septic shock are culture-proven BSIs, for which there is a rapid decline in survival rates in cases where inadequate antimicrobial therapy is administered within the first 24 hours [3,4]. As a consequence, clinicians often resort to empirical broad-spectrum antimicrobial therapy favoring the selection and spread of resistant pathogens (i.e., extended spectrum β-lactamase producing organisms) [5], increased invasive fungal infections [6,7], rising consumption of more expensive third-line drugs (e.g. tigecycline, daptomycine) [8] and drug toxicity issues (e.g. colistin) [9,10].

Rapid detection of BSIs with determination of antibiotic susceptibility can alter current practices in infection control, therapeutic management, and clinical decision-making and ultimately reduce over-prescription of antimicrobials and associated adverse outcomes [11,12]. This article will present recent technological developments, discuss the shortcomings and advantages of culture-based and molecular diagnostics and describe the requirements for an ideal rapid diagnostic system.

Definitions

Primary BSI refers to intravascular catheter-related BSI and BSI without an identifiable focus of infection while secondary BSI refers to patients infected by microorganisms originating from another site [13]. The definition of BSI poses certain diagnostic challenges as it coincides with conditions such as transient, intermittent and continuous low-grade bacteraemia. Transient bacteraemia, lasting from minutes to hours, can occur during procedures involving anatomic sites colonized by normal microbial flora (for example, tooth brushing, colonoscopy, dental extractions) or after manipulation of localized skin infections (for example, furuncles). Intermittent bacteraemia, typically associated with closed-space or focal infections (for example, abscesses, pneumonia, osteomyelitis), is characterized by recurrent episodes of bacteraemia because of cyclical clearance and recurrence of the same pathogen. Continuous low-grade bacteraemia is commonly associated with an intravascular focus of infection such as infective endocarditis or vascular-graft infections [14,15]. These definitions pose interpretational challenges and should always be put in the context of the clinical presentation as the presence of microorganisms in the bloodstream may merely represent a route of transportation rather than evidence of true BSI. This could occur not only in cases of transient bacteraemia but also when the source of infection is outside the bloodstream.

Blood culture-based detection of bloodstream infections

Blood cultures are still considered the gold standard of BSI diagnosis, retrieving viable microorganisms to

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determine the species and their antimicrobial susceptibility. Blood cultures are capable of detecting as low as 1 colony-forming unit (CFU) of bacteria or fungi/yeasts per 10 ml blood. However, cultures require incubation times of up to 96 hours, can only detect cultivable microorganisms and have low sensitivity for slow growing, intracellular and fastidious microorganisms and in patients pre-treated with antimicrobials [16]. The positive predictive value of cultures is estimated to be above 95% but in BSI the overall positivity may be as low as 30 to 40% despite proper implementation of standard procedures, adequate blood volume collection and substantial clinical suspicion of BSI [16-18].

Collection of two to three blood culture sets per suspected BSI episode with 20 to 30 ml of blood per set (an aerobic and anaerobic bottle) in adults is advised as more sets and higher volumes of blood correspond with a higher detection rate [19]; however, extracting such volumes of blood is not feasible in children and neonates. Furthermore, 50% of blood cultures may have inadequate volumes of blood, which may ultimately reduce the overall sensitivity of blood cultures [20]. In case of clinically significant bacteraemia, the number of recoverable bacteria in blood of adults is reported to be in the range of 1 to 30 CFU/ml, while it may exceed 100 CFU/ml in children [21].

Detection time has been significantly reduced by recent advances, including the development of new liquid media and growth supplements, the development of adsorbing agents to neutralize growth inhibitors, metabolic products and remnant antibiotics, the introduction of fully automated instruments with continuous monitoring of growth based on analysis of the proportional release of CO_2 using fluorescent or colorimetric sensors, and the co-application of new molecular technologies [22]. Nevertheless, the median time to positivity of blood cultures is still 15 hours (range, 2.6 to 127 hours), while the total time until full identification, including antimicrobial susceptibility, is often more than 72 hours for bacteria and more than 60 hours for fungi [22-24]. The time to positivity of blood cultures depends on the severity of the disease, the bacterial/fungal load, the type of pathogen and its growth capacity, polymicrobial infection, the volume of cultured blood, the system used for blood culturing, the time from sampling to incubation, and last but not least the presence of growth suppressants (for example, antibiotics) [22-24]. Thus, blood cultures are far from being an ideal gold standard as results are often delayed and incomplete and may not reflect all bacteriological evidence.

Nucleic acid testing

The application and assimilation of nucleic acid testing (NAT) for diagnosis of BSI have been hampered by recent improvements in culture-based methods and the disadvantage of representing add-on techniques that do not replace conventional blood cultures. Most of the NATs introduced within the past 20 years have been complex technologies with too small platforms, and are often poorly standardized tests developed in-house with limited impact on clinical decision-making. In addition, they may be more labour intensive and expensive and sometimes even less sensitive than culture-based approaches. The existing commercial NATs are, in general, based on a similar paradigm: pathogen lysis; nucleic acid extraction and purification; amplification of nucleic acids by PCR; and identification method (for example, ELISA-based hybridization, fluorescence-based real time detection, liquid or solid phase microarray detection, sequencing and database recognition).

In general, BSI assays either require prior cultivation steps and initiation from positive blood cultures or single colonies or are assays directly applicable to blood specimens. Irrespective of the need for prior cultivation, there are three main methodological approaches: single pathogen-specific assays that target species- or genus-specific genes (for example, PCR or other amplification-based assays, hybridisation or fluorescence *in situ* hybridization); universal broad range assays targeting specific sequences in the bacterial or fungal genome (for example, panbacterial 16S, 5S, 23S rDNA/RNA or panfungal 8S,18S, 5.8S, 28S rDNAs/RNAs) in blood followed by pathogen-specific identification; and multiplex PCR assays for parallel detection of species- or genus-specific targets of different pathogens. Tables 1 and 2 provide an overview of the most important, commercially available devices.

Molecular identification of pathogens based on positive blood cultures

Various culture-based NATs are currently commercially available. The most important limitation of these assays, however, is that they do not overcome the technical and sensitivity issues of blood cultures, cannot be directly applied to other biological specimens and are often only partially faster than non molecular-based technologies. Pathogen-specific assays are capable of detecting genes encoding resistance to antimicrobials, such as *mecA* in staphylococci or *van* genes in enterococci, allowing a faster phenotypic detection of resistance. These assays may have added value for antibiotic stewardship decisions by allowing the rapid de-escalation of empiric broad-spectrum antibiotic treatment [25].

One such assay approved by the US Food and Drug Administration (FDA) is the GeneXpert system (Cepheid, Sunnyvale, CA, USA), which combines sample preparation and detection with real-time PCR in a closed and compact cartridge. GeneXpert detects methicillin

Table 1. Current molecular techniques for detection of bloodstream infection, performed on positive blood cultures

Assay/ manufacturer	Technique	Anti-microbial resistance genes	Detectable pathogens	Detection limit (CFU/ml)	Turn-around time (h)	Costs equipment/ test and supplies	Sensitivity/ specificity
PNA-FISH (AdvanDX, MA, USA)	Fluorescence-based hybridization with PNA probes	None	*S. aureus*, CoNS, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *C. tropicalis*	NA	1.5-3	$/++	>98%/>98%
AccuProbe [104,105] (Gen-Probe Inc., CA, USA)	Chemi-luminescent DNA probes detect rRNA	None	*Streptococcus pneumoniae*, enterococci, streptococci group A and B	NA	2.5	$$$/+++	72%/>99%
Nanosphere Verigene BC-GP	Multiplex PCR	*mecA*, *vanA*, *vanB*	*S. aureus*, *S. epidermidis*, *S. lugdunensis*, *S. anginosus* group, *S. agalactiae*, *S. pyogenes*, *Enterococcus faecalis*, *E. faecium*, *Listeria* spp., *Micrococcus* spp.	NA	2.5	$$/++	>98%/>98%
MALDI-TOF (bioMérieux, France or Bruker Daltonics, Germany)	Mass spectral signal recognition, laser desorption ionisation, mass spectrometry	None	Same pathogens as usually detected by blood cultures. Limitations in presence of polymicrobial bacteraemia or with some closely related organisms (for example, viridians *Streptococcus* spp., *Streptococcus pneumonia* identified as *Streptococcus parasanguinis*, *Shighella sonnei* identified as *E. coli*)	NA	<1-2	$$$/+	>76%/>96% (genus level)
Plex-ID (Abbott , USA)	Multiplex PCR detected by electrospray ionization mass spectrometry	*mecA*, *van*	Same pathogens as usually detected by blood cultures. High positive agreement with MALDI-TOF	NA	<3	$$$$/++	NA/NA
Hyplex Bloodscreen (BAG, Germany)	Multiplex PCR, hybridization on ELISA plate	*mecA*, *van*	MSSA, MRSA, *S. epidermidis*, *S. pyogenes*, *S. pneumoniae*, *E. faecium*, *E. faecalis*, *E. coli*, *E. aerogenes*, *P. aeruginosa*, *Klebsiella* spp.	NA	3	$/++	>96%/>92%
Prove-it Sepsis (Mobidiag, Finland)	Multiplex PCR + hybridization on microarray	*mecA*	CoNS, *S. aureus*, *S. epidermidis*, *S.pyogenes*, *S. agalactiae*, *S. dygalactiae ss. Equisimilis*, *S. pneumoniae*, *E. faecalis*, *E. faecium*, *Listeria monocytogenes*, *Clostridium perfringens*, *Propionibacterium acnes*, Enterobacteriaceae, *E. coli*, *K. oxytoca*, *K. pneumoniae*, *E. aerogenes*, *E. cloacae*, *P. mirabilis*, *P. vulgaris*, *Salmonella* spp., *Serratia marcescens*, *Campylobacter* spp., *H. influenzae*, *Neisseria* spp., *Kingella kingae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Bacteroides fragilis*,	NA	3	$/++	>94%/98%
Black-Light (BlackBio, Spain)	Broad-range PCR and pyrosequencing	NA	Multiple sepsis pathogens, including *Candida* spp., *Aspergillus fumigatus*, *Aspergillus niger*	NA	4	$$$/++	NA/NA
StaphPlex system (Qiagen, CA, USA)	Multiplex PCR + lbead-based suspension array	*Tuf*, *nuc*, *meqA*, *aacA*, *ermA*, *ermC*, *tetM*, *tetK*	18 *Staphylococcus* genes (*S. aureus* and most clinically relevant coagulase negative staphylococci)	NA	5	$$$/++	72-100%/>95%
Staph SR (BD GeneOhm, CA, USA)	Real-time multiplex PCR	*mecA*	*S. aureus* (MRSA/MSSA detection)	NA	2.5-3	$$/++	>95%/>85%
Gene Xpert MRSA/MSSA (Cepheid, CA, USA)	Real-time multiplex PCR	*SCCmec*, *mecA*	*S. aureus* (MRSA/MSSA detection)	NA	1	$$$/+++	>98%/>98%
KeyPath (Microphage, USA)	Real-time pathogen- specific PCR	*mecA*, *nuc*	*S. aureus* (MRSA/MSSA detection)	NA	<5	$$/++	100%/100%

$, <35,000 dollars; $$, <75,000 dollars; $$$, <200,000 dollars; $$$$, >200,000 dollars; +, <10 dollars; ++, 10 to 50 dollars; +++, >50 dollars. CFU, colony forming unit; CoNS, coagulase-negative staphylococci; ELISA, enzyme-linked immunosorbent assay; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MRSA, methicillin resistant Staphylococcus aureus; MSSA, methicillin-sensitive Staphylococcus aureus; NA, not available; PCR, Polymerase chain reaction; PNA-FISH, peptide nucleic acid-fluorescence *in situ* hybridization.

Table 2. Current molecular techniques for detection of bloodstream infection, performed directly on whole blood

Assay/ manufacturer	Technique	Anti-microbial resistance genes	Detectable pathogens	Detection limit (CFU/ml)	Turn-around time (h)	Costs equipment/ test and supplies	Sensitivity/ specificity
SepsiTest (Molzym, Germany)	Broad-range PCR + sequencing	None	>300 different pathogens	20-40 for *S. aureus*	8-12	$$$/+++	82%/86-90%
Vyoo (SIRS-Lab, Germany)	Multiplex PCR + gel electrophoresis	*mec*, *vanA*, *vanB*, *vanC*, bla_{SHV}	*S. pyogenes*, *S. pneumoniae*, *S. agalactiae*, viridans-group streptococci species, *S. aureus*, CoNS, *E. faecalis*, *E. faecium*, *Clostridium perfringens*, *Bacillus cereus*, *E. coli*, *E. aerogenes*, *E. cloacae*, *K. oxytoca*, *K. pneumoniae*, *P. mirabilis*, *S. marcescens*, *Morganella morganii*, *P. aeruginosa*, *S. maltophilia*, *A. baumannii*, *Burkholderia cepacia*, *H. influenzae*, *Neisseria meningitidis*, *Bacteroides fragilis*, *Prevotella buccae*, *P. melaninogenica*, *P. intermedia*, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *Aspergillus fumigatus*	3-10	8	$$$/++	NA/NA
LightCycler SeptiFast (Roche, Germany)	Multiplex real-time PCR	*mecA*	*S. aureus*, CoNS, *Streptococcus pneumoniae*, *Streptococcus* spp. (*S. pyogenes*, *S. agalactiae*, *S. mitis*), *E. faecium*, *E. faecalis*, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Proteus mirabilis*, *E. cloacae*, *E. aerogenes*, *Serratia marcescens*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *Aspergillus fumigatus*	3-30	6	$$/+++	60-95%/74-99%[a]
Plex-ID (Abbott , USA)	Multiplex PCR detected by electrospray ionization mass spectrometry	*mecA*, *vanA*, *vanB*, *vanC*, bla_{KPC}	Multiple pathogens and bioagents, including *Yersinia pestis*, *Bacillus anthracis*. Separate dedicated assays for viruses or yeasts/fungi	NA	8-12	$$$$/++	NA/NA
VAPChip [106] (Eppendorf Array Technologies, Belgium)	Multiplex PCR + hybridization on a cartridge-containing microarray	bla_{TEM}, bla_{SHV}, bla_{CTX-M}, carbapenemases[b], *mecA*	*S. aureus*, *S. pneumoniae*, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *E. cloacae*, *E. aerogenes*, *S. marcescens*, *A. baumannii*, *P. aeruginosa*, *S. maltophilia*, *Haemophilus influenzae*, *Legionella pneumophila*	>10	5-8	NA	NA/NA
MagicPlex (SeeGene, Korea)	Multiplex real-time PCR	*vanA*, *VanB*, *mecA*	73 Gram-positive (30 *Staphylococcus* spp., 40 *Streptococcus* spp., 3 *Enterococcus* spp.), *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Serratia marcescens*, *Bacteroides fragilis*, *Salmonella typhi*, *Klebsiella oxytoca*, *Proteus mirabilis*, *E. coli*, *E. cloacae*, *E. aerogenes*, *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, *Aspergillus fumigatus*. Separate dedicated assays for viruses	NA	3-4	NA	NA/NA

[a]Sensitivity and specificity varies depending on the pathogen of interest. [b]7 OXA-23, 6 OXA-58, 5 OXA-48, 5 VIM-2, 4 VIM-4, 3 KPC-2, 3 IMP-13, 2 VIM-1, 2 OXA-72, 1 VIM-like, 1, IMP-4, 1 OXA-40. $, <35,000 dollars; $$, <75,000 dollars; $$$, <200,000 dollars; $$$$, >200,000 dollars; +, <10 dollars; ++, 10 to 50 dollars; +++, >50 dollars. CFU, colony forming unit; CoNS, coagulase-negative staphylococci; ELISA, enzyme-linked immunosorbent assay; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MRSA, methicillin resistant Staphylococcus aureus; MSSA, methicillin-sensitive Staphylococcus aureus; NA, not available; PCR, Polymerase chain reaction; PNA-FISH, peptide nucleic acid-fluorescence *in situ* hybridization.

resistance or susceptibility (MRSA/MSSA) based on sequences in staphylococcal protein A (*spa*), *SCCmec* and *mecA* genes and has a turnaround time of 1 hour. It has a reported sensitivity of 100% and a specificity of 98.6% for *Staphylococcus aureus* detection and 98.3% and 99.4% for MRSA detection, respectively [26-28]. However, there is a small risk of false positives when testing for the *SCCmec* gene in the presence of methicillin-resistant, coagulase-negative *Staphylococcus* (CoNS) spp.

Another recently FDA-approved assay capable of identifying MRSA/MSSA within 5 hours based on evaluation of *mecA* and *nuc* genes is the KeyPath system (MicroPhage, Longmont, CO, USA) [29]. Another FDA-approved assay is StaphSR (BD GeneOhm, La Jolla, CA, USA), which runs on the SmartCycler instrument and amplifies specific target sequences of *S. aureus* for rapid detection of MSSA/MRSA with a turnaround time of 2.5 hours. For MRSA, the sensitivity, specificity, and positive and negative predictive values are reported to be 95.9%, 85.3%, 58.5%, and 99.0%, respectively [30,31]. However, other studies have shown discrepant results from this assay due to the presence of borderline MRSA strains [32,33].

Broad-range assays require an initial PCR amplification of a target sequence followed by several further identification strategies, such as sequencing, fragment size analysis or hybridization. Alternatively, the analysis of polymorphism or subsequent genus or species identification can be performed by real-time PCR assays. They target conserved sequences of bacterial and fungal genes such as those encoding ribosomal DNA.

Prove-it Sepsis (Mobidiag, Helsinki, Finland) is one of the first commercially available microarray-based molecular multiplex assays for sepsis detection in blood cultures. It combines broad-range PCR with amplification of *gyrB*, *ParE* and *mecA* genes. It uses the Prove-it™ StripArray system, which is compatible with its tube-array platform, and has a larger detection panel for both Gram-positive and Gram-negative bacteria than other existing culture-based NAT methods (24 species, 24 taxons, *mecA* for MRSA) with a turnaround time of 3 hours and a reported sensitivity of 95% and specificity of 99% for the pathogens included in its panel. It covers around 90% of all sepsis-causing pathogens, including fungi, but is a labour- and expertise-demanding approach [34].

BlackLight (BlackBio, Madrid, Spain) is a recent broad-range assay that identifies various bacteria by covering three regions of the 16S ribosomal gene with a turnaround time of 4 hours. BlackLight detects *Candida* spp., *Aspergillus fumigatus* and *Aspergillus niger* and more than 400 fungal and yeast sequences based on the 18S rRNA. It is a multistep approach based on pre-amplification reactions, including gelified PCR, three simultaneously conducted amplification reactions, three-step pyrosequencing and final identification by comparison with an existing database [35]. Clinical validation is still pending.

Multiplex assays with real-time PCR target different genes of pathogens identified from blood cultures in a single reaction but do not allow a direct application to blood samples. Hyplex BloodScreen (BAG, Lich, Germany) is a multiplex PCR assay that is able to detect several bacterial species via hybridisation in an ELISA-like format. It has a turnaround time of 3 to 4 hours, sensitivity >96% and specificity >92% and detects various resistance markers such as mecA, *van* and several β-lactamase genes [36,37].

StaphPlex (Qiagen, Valencia, CA, USA) is a culture-based multiplex PCR designed to identify *S. aureus* and several CoNS with various corresponding antimicrobial resistance determinants by combining the amplification of multiple targets (Biotin label) followed by hybridisation of the biotinylated PCR products on specific colour-coded capture probes with the Luminex xMAP technology [38,39]. The turnaround time is around 5 hours, with sensitivities ranging between 72.5% and 100% depending on the staphylococcal species identified.

The Nanosphere Verigene BC-GP system (Nanonsphere, Northbrook, IL, USA) is a recently CE-approved multiplex Gram-positive blood culture-based device capable of providing information on genus, species, and resistance detection of various pathogens within 2.5 hours on the same automated platform (Table 1) [40]. It provides information on resistance markers (*mecA*, *vanA* and *vanB*). A similar test for Gram-negative bacteria is expected to be launched soon. This platform still needs further clinical validation.

Beyond the scope of the amplification-based methods, peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH; AdvanDx, Woburn, MA, USA) is an interesting alternative approach for antimicrobial stewardship. PNA probes, which are synthetic oligomers mimicking the DNA or RNA structure, allow the detection of microorganisms without the need for an amplification step and are thus less likely to be affected by contamination [40]. PNA-FISH enables targeting the rDNA of several *Candida* species and rRNA of a few bacteria. It does not require expensive infrastructure, is suitable for settings with lower resources, has a turnaround time of 2 to 3 hours and has an excellent sensitivity and specificity according to manufacturer-sponsored studies [41-46]. However, PNA-FISH has an insufficient detection panel for pathogens, is labour intensive, relies on previous Gram stain results and, most importantly, requires experienced and skilled technicians to interpret the results [42-44]. Furthermore, it requires active support from an antimicrobial stewardship team, otherwise there may be no added diagnostic value [45,46].

One of the greatest progresses in rapid detection of BSI-causing pathogens is related to protein-based identification via mass spectrometry (MS) techniques. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS (Bruker Daltonics, Bremen, Germany or BioMérieux, Marcy l'Etoile, France) performed on colonies obtained from culture-positive specimens, or even directly from aliquots of positive blood culture bottles, compares the mass spectral signals with a database of spectra from reference standard spectra. It is very swift (turnaround time of 1 to 2 hours), accurate (sensitivity 76 to 98% depending on the pathogen, specificity >96%) and reduces labour load. However, Gram-negative organisms appear to be much easier to detect than Gram-positives, with *Streptococcus* and *Staphylococcus* species and most commonly *Streptococcus pneumoniae* causing certain challenges [47-49]. It has a high device purchase cost (around 150,000 euros) but is rather inexpensive to use. However, MALDI-TOF MS does not reduce the required time for antibiotic resistance testing and still requires positive cultures. Furthermore, it fails to accurately identify polymicrobial infections due to dynamic range issues in the mass spectrometer and often detects only one pathogen of interest or may even cause false species identification [47-51].

Direct pathogen identification by nucleic acid testing

During the past 20 years, several PCR assays capable of direct detection of pathogens in blood and cerebrospinal fluid without prior cultivation have been introduced. Several automated platforms, either commercially available or under development, have the aim of direct detection of bacteria or fungi in blood components. Similar to culture-based methods, NAT assays can be divided between pathogen/genus-specific, broad-range and multiplex assays.

Several pathogen-specific assays have been developed for specific molecular targets and performed on specimens such as EDTA blood samples, serum, cardiac valves and vascular biopsy specimens [15,52-54]. They may be useful in cases of patients with a history of culture-negative infections caused by fastidious or slow-growing microorganisms. Genus-specific assays detect only a group of pathogens without detailed identification of the species, for instance, invasive aspergillosis in bronchoalveolar lavage fluid [55].

Broad-range assays potentially allow direct detection of cultivable and non-cultivable pathogens. The existing methods combine universal PCR, targeting conserved regions with sequencing, hybridisation or electrospray ionisation (ESI) MS. SeptiTest (Molzym, Bremen, Germany) is the only commercially approved broad-range PCR assay developed for diagnosis of BSI directly on blood without previous culturing. It targets 16S rRNA genes of bacteria and the 18S rDNA of fungi and contains a four-step approach (DNA extraction, PCR amplification, sequencing using primers, online-identification), which reduces issues caused by the presence of human DNA in an extracted specimen [56-58]. However, SeptiTest requires careful handling, is expensive, has a turnaround time of 5 to 12 hours and a high risk of false positives due to the multistep approach.

Multiplex real-time PCR assays and microarrays amplify multiple targets of DNA in the same biological sample simultaneously and is the most promising approach. However, the commercially available methods still lack sensitivity and a sufficiently broad panel of antibiotic resistance markers. The most commonly used device available since 2004, SeptiFast (Roche, Mannheim, Germany) is a real-time multiplex PCR-based assay available for sepsis diagnosis that can detect 25 clinically important bacteria and fungi directly from whole blood in about 6 hours [59-61]. SeptiFast is by far the most studied and validated assay and has a reported range of 60 to 95% sensitivity and 74 to 99% specificity depending on the target pathogen [59-72]. However, it is very labour-intensive, requires expertise to use, and has a high cost (150 to 200 euros per test). Moreover, except for MRSA, it provides no information on antimicrobial susceptibility. In a recent cohort study from Japan [73], it was shown that SeptiFast could complement traditional culture-based methods, particularly in antibiotic-treated patients.

VYOO (LOOXSTER, SIRS-Lab, Jena, Germany) is a multistep multiplex PCR (mechanical lysis and purification of DNA, human background DNA removal, PCR amplification of target DNA, amplicon gel electrophoresis and analysis) that can potentially detect 35 bacterial and 6 fungal species and important antimicrobial resistance detection, such as bla-*SHV β-lactamase*, *mecA* and *vanA*, *B*, and *C* genes (vancomycin resistance) [57,74,75]. VYOO requires prior selective enrichment of microbial DNA (removal of human DNA) in a mixture with excess eukaryotic DNA by affinity chromatography, which may increase sensitivity to 3 to 10 CFU/ml. The overall turnaround time is about 6 to 8 hours, but is not easy to use and has been clinically insufficiently validated.

PLEX-ID (Abbott Molecular, Carlsbad, CA, USA) is a recent multiplex real-time automated PCR with amplicon product detection by ESI MS [76,77]. PCR-ESI MS is designed to rapidly identify genotypic characterization of a broad range of bacteria, fungi, viruses and parasites in a given sample (for example, cultures or whole blood) via broad-range primers specific for groups of pathogens rather than for any one particular species [76,77]. Additional primer-pairs target identifiable genes for

antibiotic resistance or particular pathogenic features [78,79]. PLEX-ID can identify more than one microorganism per sample, provides quantitative assessment of pathogen load, and detects drug resistance marker genes such as *mecA*, *vanA*, *vanB* and bla_{KPC} (carbapenem class in *Klebsiella pneumoniae* and other Gram-negative bacilli) [80-82]. A recent study indicates very good agreement between PCR-ESI MS and MALDI-TOF MS in detecting pathogens at the genus and species levels (0.94 to 0.97) [83]. It is currently not available for clinical diagnostics due to the very high cost and undetermined sensitivity; clinical trials are pending.

MagicPlex (SeeGene, Seoul, Korea) is the most recent multiplex device with several platforms, including Magicplex Sepsis, which is able to detect more than 73 Gram-positive and 12 Gram-negative bacteria, 3 drug resistance markers (*mecA*, *vanA* and *vanB*) and 6 fungi, covering over 90% of sepsis-related microorganisms. It is a multistep approach, where pathogen amplification is carried out by initially using the SelectNA™ blood pathogen kit, which extracts pathogen DNA from 1 ml of whole-blood via an automated magnetic bead nucleic acid device (SEEPREP12™). It is then followed by a pathogen amplification and detection process, combining conventional PCR (SEEAMP™) with real-time PCR (for example, CFX96™, Bio-Rad; SmartCycler, Cepheid). It provides final information within 6 hours but has not yet been clinically validated [84].

Limitation of nucleic acid testing

The evaluation of NAT-based technologies for BSI diagnosis should encompass issues such as the range of detection, access to additional information such as antimicrobial susceptibility, turnaround time, throughput, technical complexity, time and effort required, as well as overall costs. More importantly, major issues to be addressed are differentiation of viable from non-viable microorganisms and the issue of microbial DNAaemia (DNA from live microorganisms versus DNA from dead microorganisms or free circulating DNA versus phagocytised DNA in immuno-competent host cells) as markers for an infectious focus somewhere in the host. The latter should be in the context of consideration and interpretation of the risk of potential contamination, as in the case of Gram-positive bacteria for blood cultures.

To date, the published clinical evaluation studies have suffered from serious shortcomings, such as the application of an inappropriate gold standard (blood cultures); emphasis on microbiological rather than clinical evaluation; no convincing cost-effectiveness analysis; no guidance for targeting appropriate clinical situations; and the potential for wrong interpretation of results if no expert assistance and advice are available. Thus, application of NAT in routine clinical practice requires more sophisticated infectious disease knowledge and guidance since the results may potentially complicate clinical decision making.

Pathogen-specific technologies often have specificity to one or a limited group of pathogens and may require add-on tests in order to exclude specific pathogens such as MRSA before enabling the clinician to narrow empiric antibiotic treatment. Broad-range NAT assays are better suited to detect pathogens identified by blood culture, but they are labour-intensive and costly, have low sensitivity and specificity due to the multi-step approach (risk of contamination), and save only marginal time compared to techniques such as MALDI-TOF MS when performed directly from positive blood cultures [85]. The risk of contamination refers not only to laboratory procedures during the PCR process (which has been dramatically reduced by closed systems) but also to contamination when obtaining the blood sample.

As new NATs are compared with blood cultures for diagnosis of BSI, a positive result for bacterial or fungal nucleic acids in the presence of negative culture poses interpretational challenges. It may reflect detection of pathogens due to a higher sensitivity of NAT for certain slow-growing or fastidious organisms. But NAT may also detect circulating nucleic acids, or non-proliferating, dead or degraded pathogens, which may be of clinical importance, for example, in antibiotic pre-treated patients in the ICU (for example, meningococcal sepsis). Finally, it may reflect contamination (for example, by CoNS) or carryover nucleic acid, especially after successful antimicrobial therapy, which may be detectable up to several days without apparent clinical significance [86,87].

Despite the potentially high positive predictive value of the current NATs, the negative predictive value may be insufficient to exclude infection and the majority of the existing technologies are neither sensitive nor specific enough. Thus, more research is needed to improve the interpretation of circulating microbial nucleic acids (DNAaemia).

Finally, despite manufacturers' declared short turnaround times, clinicians often report a different experience when it comes to the availability of results. Under real-life conditions there are often considerable delays due to practical issues, such as specimen transportation and batch-wise analysis of samples, which reduce the intrinsic advantage of shorter turnaround times of the amplification-based tests, and availability of staff outside daily routines. In a recent study, for instance, the median time for availability of a SeptiFast result was between 18 and 27 hours [88], which contradicts recently reported potential cost and survival gains using SeptiFast for pathogen detection in sepsis [89].

Criteria and challenges for improved point-of-care diagnostic tools for detection of BSI

Development of the 'perfect' rapid diagnostic test for BSI hinges on two issues, sample preparation and detection. Sample preparation, including collection, lysis, extraction and enrichment of pathogen nucleic acid, is challenging since the microbial load in a sample can be as low as 0.1 CFU/ml in blood [90,91]. Thus, accurate diagnosis requires volumes of around 10 ml, but the nucleic acid loads of such volumes of blood will far exceed the purification capacity of current microfluid technologies as well as their overall volume capacity (<1 ml) [92]. Bacteraemia in adults often presents with less than 10 CFU/ml (range of 1 to 30 CFU/ml), which is below the level of analytical sensitivity of many current PCR assays (3 to 100 CFU/ml) [93].

A whole blood specimen contains human DNA located in circulating white blood cells, which will be co-isolated in great excess compared to pathogen nucleic acid and thus will decrease the sensitivity of pathogen detection [88]. Inadequate breakdown of distinct pathogens that are difficult to lyse and prepare adequately during nucleic acid extraction (for example, Gram-positive, yeasts, molds) is a further limitation [91,92]. Additionally, pathogens can, in certain cases, be adherent or sequestrated in blood cells and platelets, which may hamper the initial separation and extraction of pathogen nucleic acids [94].

As new NATs converge towards the principle of lab-on-chip systems, most of the existing platforms are thus considered inadequate due to the requirement of pre-enrichment of the specimen, the reliance on prior cultivation in blood cultures and the limited antibiotic resistance testing. Although it is relatively simple to design strategies for single gene encoded resistance detection (for example, the *mecA* gene for methicillin resistance in *S. aureus*), this is not the case for many bacterial resistance types based on several distinct point mutations, such as penicillin resistance in pneumococci, and for more than 300 extended-spectrum β-lactamases among Gram-negative species [91,95].

Additionally, phenotypic resistance is influenced by different regulatory genes that play an important role in the expression of genes determining antimicrobial resistance. In the case of Gram-negative bacteria, the resistance genes are constantly evolving, making it costly and technically challenging to integrate the clinically most important genes into the existing or future NAT platforms. This reduces the negative predictive value of such assays, which is crucial in the clinical decision-making process, since clinicians need very high negative predictive values (ideally >98%) in order not to overuse broad spectrum antimicrobials such as carbapenems or not systematically add colistin, tigecyline or amikacin in settings with hyperendemic carbapenemase-producing bacteria.

Future assays require fully automated strategies applicable to various specimens that are also capable of quantifying the pathogen load as either a marker for disease severity (for example, *S. pneumoniae*) or in order to distinguish contamination (for example, CoNS) [96]. They should ideally provide relevant information 2 to 6 hours after specimens are taken in order to have an impact on the choice of treatment. They also need to improve the limited analytical sensitivity for clinically relevant low bacterial loads and for detection of certain bacteria and fungi that are difficult to detect, to distinguish between living and dead bacteria, to evaluate the role of transient bacteriaemia/fungaemia when no clinically apparent signs of infection are present, and finally to evaluate the impact of DNAaemia (DNA footprint) in cases of clinical signs of BSI.

Conclusion

Direct detection and identification of pathogens in blood or other specimens for rapid diagnosis of BSI by molecular approaches is a promising idea since it will facilitate early appropriate pathogen-driven therapy [97]. Despite substantial technological advances in the past decade, there is still a need for automated selective enrichment procedures for bacterial and fungal nucleic acids, blocking or elimination methods to eliminate excess human DNA, and use of viability markers to identify clinically relevant findings [22,91]. The target population most likely to benefit from the introduction of the rapid BSI diagnosis includes children (higher bacterial loads) [98] and patients with a high risk for infections with slow-growing, non-cultivable or intracellular bacteria or fungi, for example, neutropenic, transplant, critically ill and particularly antibiotic-pretreated patients.

An adequately validated NAT may ultimately influence trial design and facilitate the introduction of new antimicrobials by enabling more feasible comparative clinical trials (selected pathogens such as extended-spectrum β-lactamase producers, smaller sample size, substantial decrease in overall cost) and potentially assist in the introduction of new drugs.

The number of commercial PCR platforms for detection of pathogens is rapidly increasing but the lack of methodological and scientific rigor in the clinical examination and approval of emerging medical devices and failures in regulatory oversight before widespread clinical use, particularly in Europe [99,100], are worrisome. There is a lack of transparency in publishing research findings, device-related complications, adverse outcomes, and competing interests, since no formal and specific requirements for clinical evaluation trials of new molecular BSI test systems have been provided despite the introduction of *The Standards for Reporting Diagnostic Accuracy* [99,100] and studies suggesting that new diagnostic tests

should be more rigorously evaluated to establish their added value for clinical decision-making [101]. This stands in contrast to the many regulatory hurdles that companies face before introducing new antimicrobial compounds onto the market. Furthermore, the clinical and regulatory assessment of molecular BSI diagnosis systems should require a different reference approach based on clinical, radiological and bacteriological data, since blood cultures remain an imperfect gold standard due to inadequacy of blood cultures to detect many true cases of infection.

Currently, no available molecular method is, by itself, sufficiently accurate or sensitive enough to replace methods based on blood culture. Additionally, they tend to be more expensive and time-consuming to a degree which disqualifies them from being truly rapid diagnostics and thus limits them to certain expert labs.

Nevertheless, there is some evidence to support the add-on value of existing NATs as an adjunct to current culture methods in facilitating not only the early detection of more microorganisms (true positives) and important resistance genes (for example, *mecA*) but also in reducing the time to optimal antimicrobial therapy and subsequent improved endpoints, such as reduced mortality, length of stay in hospital, overall costs and the use of broad-spectrum antibiotics [27,28,43,44, 72,89,102]. Direct detection of pathogenic nucleic acids without prior cultivation despite diverging results from culture-based methods together with possible information on resistance markers may have significant added value in enabling evidence-based antibiotic stewardship, as detection of nucleic acid may indeed reflect the true clinical picture in specific clinical situations [103].

Continuous advances in whole-genome sequencing methods have the potential to enable parallel sequencing of pathogen specimens with various antimicrobial resistance elements. This will potentially guide treatment based solely on molecular measurements and subsequently eliminate the need for culture methods. However, this requires further improvements in areas such as interpretation of results (because of the inevitable increase in the detection of inconsequential nucleic acids from transient shedding and dead organisms), data management, verification and validation of sequence databases, cost of instrumentation and user friendliness.

A realistic, pragmatic and near-term solution that could potentially improve antibiotic stewardship would be the development of a rapid real-time nucleic acid point-of-care test that detects few but clinically relevant pathogens (*P. aeruginosa*, *A. baumanii*, *S. aureus*, *Klebsiella* spp., *E. coli* and *Candida* spp.) and resistance genes (*KPC*, *NDM*, *OXA 48*, *mecA*, *vanA* and *vanB*). Ultimately, rapid molecular BSI diagnostics have the potential to become an essential tool for antibiotic stewardship in ICUs.

Abbreviations

BSI, bloodstream infection; CFU, colony-forming unit; CoNS, coagulase-negative staphylococci; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FDA, Food and Drug Administration; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight; MRSA, methicillin-resistant *Staphylococcus aureus*; MS, mass spectrometry; MSSA, methicillin-sensitive *Staphylococcus aureus*; NAT, nucleic acid test/testing; PCR, polymerase chain reaction; PNA, peptide nucleic acid; PNA-FISH, peptide nucleic acid-fluorescence in situ hybridization.

Competing interests

SH has received consultant and speaker honoraria from bioMérieux (Marcy l'Etoile, France), Da Volterra (Paris, France), and Destiny Pharma (Brighton, UK). JS is Chief Medical Advisor for bioMérieux. AA and MI declare that they have no competing interests.

Authors' statement

All authors state that they have read and approved the manuscript. It has not been published nor is it under consideration for publication elsewhere.

Acknowledgments

Work by the authors was supported by the Innovative Medicines Initiative, a public-private partnership between the European Union and the European Federation of Pharmaceutical Industries and Associations (Rapp-ID grant agreement, no. 115153).

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Published: 29 May 2012

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doi:10.1186/cc11202
Cite this article as: Afshari A, *et al.*: Bench-to-bedside review: Rapid molecular diagnostics for bloodstream infection - a new frontier? *Critical Care* 2012, **16:**222.