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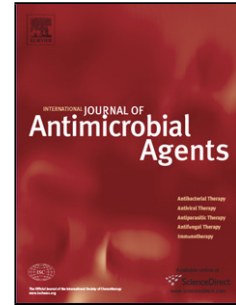
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**Conventional and molecular techniques for the early diagnosis
of bacteraemia**

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Abstract

Bloodstream infections account for 30–40% of all cases of severe sepsis and septic shock, and are major causes of morbidity and mortality. Diagnosis of bloodstream infections must be performed promptly so that adequate antimicrobial therapy can be started and patient outcome improved. An ideal diagnostic technology would identify the infecting organism(s) and their determinants of antibiotic resistance, in a timely manner, so that appropriate pathogen-driven therapy could begin promptly. Unfortunately, despite the essential information it provides, blood culture, the gold standard, largely fails in this purpose because time is lost waiting for bacterial or fungal growth. Several efforts have been made to optimize the performance of blood culture, such as the development of technologies to obtain rapid detection of microorganism(s) directly in blood samples or in a positive blood culture. The ideal molecular method would analyze a patient's blood sample and provide all the information needed to immediately direct optimal antimicrobial therapy for bacterial or fungal infections. Furthermore, it would provide data to assess the effectiveness of the therapy by measuring the clearance of microbial nucleic acids from the blood over time. None of the currently available molecular methods is sufficiently rapid, accurate or informative to achieve this. This review examines the principal advantages and limitations of some traditional and molecular methods commercially available to help the microbiologist and the clinician in the management of bloodstream infections.

Keywords:

Bloodstream infection

Blood culture

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1. Introduction

The definition of bacteraemia is the presence of bacteria in the bloodstream, but does not include the important clinical description of the site or source of infection [1]. The US Food and Drug Administration (FDA) guidelines prefer the term bloodstream infection (BSI) rather than bacteraemia, because the former emphasizes the need for a diagnosis based on clinical and microbiological criteria [2].

According to the National Strategy to Address Health Care Associated Infections, developed by the Australian Council for Safety and Quality in Healthcare, the National Nosocomial Infections Surveillance System (NNIS) of the US Centers for Disease Control and Prevention (CDC), and the UK Public Health Laboratory Service (PHLS) the definition of BSI in adults must meet one of the criteria listed in Table 1.

[Table 1 here]

Conventionally, BSIs have been divided into two categories: 1) primary BSIs, comprising BSIs of unknown origin in patients without an identifiable focus of infection, and intravascular catheter-related BSIs; and 2) secondary BSIs, defined as those caused by a microorganism related to an infection at another site.

BSIs account for 30–40% of all cases of severe sepsis and septic shock [3] and are major causes of morbidity and mortality. The true incidence of nosocomial

BSIs is unknown, but it is estimated that about 250 000 cases occur annually in the USA [4]. Some studies have reported the incidence of BSI to be around 1% in the intensive care unit (ICU) [5] and 36% in bone marrow transplant recipients [6]. The crude mortality rate has been reported to range from 12% in total hospital populations to 80% in ICU patients [7–11]. The rate of mortality directly attributable to BSIs in these populations has been estimated to be 16–40% [8,12]. Inappropriate empirical antimicrobial therapy is an important predictor of death in these patients [8,13,14].

Microbiology laboratories can offer two types of test to diagnose BSI.

Conventional techniques are based on culture of a sample in enriched broth, isolation of the pathogen after incubation and, finally, identification of the pathogen through its metabolic properties and susceptibility to antibiotics.

Because time is of the essence in preventing the evolution of BSI to severe sepsis or septic shock, molecular techniques have been developed to speed up one or more steps in the diagnostic workflow. This article analyses the characteristics and features of conventional and molecular microbiological techniques.

2. Conventional diagnostic techniques

Blood culture is the gold standard for the diagnosis of BSI [15]. From a diagnostic point of view it establishes the infectious aetiology of a patient's illness and provides samples of the organism for further antimicrobial susceptibility testing and antimicrobial therapy optimization. The importance of the latter aspect

cannot be underestimated, and several studies have demonstrated that inappropriate antimicrobial therapy is an independent risk factor for mortality in critically ill patients [16–19]. From a prognostic point of view positive blood culture results provide evidence of substantial failure of the host's defence mechanisms to contain an infection, or evidence of failure to remove, drain or otherwise adequately treat a primary infection site [20]. Considering the importance of blood cultures in the diagnosis and management of sepsis, it is prudent and relevant to understand the clinical and technical requirements and limitations of this technology.

2.1. Timing and volume of collection

Several variables in blood culture technology, especially an appropriate specimen collection technique, have been recognized as having a significant impact on the sensitivity and specificity of the test, on organism recovery rates and the turnaround time for reporting results [20].

The conventional approach with respect to the timing of blood sample collection is to collect specimens at around the time of a spike in body temperature in order to enhance the likelihood of detecting bacteraemia [16,21,22]. However, the rationale for this choice is questionable, because some authors have shown that the timing of blood sample collection makes no difference to the ability to detect bacteraemia [23].

The ability of blood culture to diagnose BSI is related to the volume of blood used and the number of samples collected. Different studies support the inoculation of

at least 20–30 ml [24–26] of adult blood into blood culture bottles because bacterial/fungal load in BSI can be very low (often <1 colony-forming unit (CFU)/ml) [27]. Moreover, one blood culture is not sufficient to predict BSI. If one blood culture is performed the percentage yield is 65.1%, if two consecutive blood cultures are drawn the percentage yield rises to 80.4% and if a third is performed the percentage yield reaches 95.7% [28].

BSIs in young children are presumed to have a much greater magnitude (often >100 CFU/ml) than bacteraemia in adults [29]. Earlier expert recommendations for blood cultures in infants and children stated a need to collect 1–2 ml for neonates, 2–3 ml for infants (aged 1–24 months), 3–5 ml for older children, and 10–20 ml for adolescents [30]. However, more recent recommendations, including those based on the results of the study by Kellogg et al., suggest that the volume of paediatric blood cultures should be based on body weight and the estimated total blood volume of the child, suggesting the collection of no more than 1% of the total blood volume for neonates, infants and young children [31,32].

2.2. Limitations

Blood cultures constitute an important epidemiological tool, with results that serve as a base for building an empirical therapeutic strategy. Up-to-date and timely knowledge of the most common pathogens identified in bloodstream infections in infection-prone hospital departments, such as intensive care and haematology, will guide the clinician in the initial treatment of a patient with fever

of unknown origin. However, the value of routine blood culture as a general diagnostic test for clinical management is limited to certain disorders because of the delay in results, its poor sensitivity for fastidious organisms and the use of antibiotics before blood samples are drawn [33].

A great limitation of blood cultures is the delay at least 2 days before results become available, with the result that changes in empirical antibiotic therapy are more frequently guided by clinical response than culture results [34,35,36]. In fact, if patients deteriorate or do not respond to initial empirical therapy (i.e. show persistent fever), physicians are likely to make an empirical change in therapy before culture results become available. Improvement in the speed of diagnosis is therefore essential to increase the clinical benefit of detecting BSI.

The sensitivity of blood cultures for slow-growing and fastidious organisms is usually quite poor. In addition, the culture diagnosis of invasive fungal infections has low sensitivity and results are not available for many days in most cases. Because these infections occur frequently in neutropenic patients, mortality from untreated infections is generally high.

A frequently encountered problem with blood cultures is that their sensitivity decreases greatly when blood samples are taken after the start of antimicrobial therapy [37,38]. Neutropenic patients who receive prophylactic antibiotics and develop fever have a high risk of bloodstream infection, but diagnosis is challenging because blood cultures remain negative in many cases [39]. To overcome the inhibitory effect of antibiotics on blood cultures, special culture

media have been developed, including resin-containing culture fluids. These can reduce the inhibitory effect of antibiotics on bacterial growth in culture, resulting in a modest increase in sensitivity.

2.3. Bacterial load determination

If available, quantitative measurements would be widely used in microbiology. Many years of research studies using quantitative microbiology on solid media have demonstrated that such measurements provide clinically valuable information. For example, bacterial load is predictive for the occurrence of complications and death [40]. However, the inability to routinely measure bacterial load has precluded a complete understanding of the value of this metric. Quantification of bacteria in blood is difficult to achieve by culture methods and is rarely practised in clinical laboratories because it requires subsequent plating on solid media rather than incubation of blood in liquid culture. The time required for liquid culture bottles to become positive provides some suggestion of bacterial load, but is a weak quantitative measure and varies with the microbe or microbes present.

Despite much effort to optimize broth composition and develop additives to inactivate inhibitory substances or enhance the growth of microorganisms, and the use of measures to prevent contamination during blood drawing, blood culture is not an ideal gold standard. The results are too late, incomplete and potentially misleading, to the point where the recommendations are to ignore them in many cases. Even so, blood culture is essential to performing

antimicrobial susceptibility testing and to confirm the presence of viable pathogens in blood.

3. Molecular diagnostic techniques

In the case of the most serious BSIs, those associated with septic shock, speed is of essential importance. Molecular techniques can give results more quickly than blood culture and are becoming more and more useful in decreasing laboratory turnaround times.

Many molecular techniques have been developed for the detection and identification of pathogens but, so far, few can be used on growth-positive blood cultures, and even fewer directly on blood. The disadvantages of analysing specimens after they have grown in culture are the time delay and the potential bias generated by a previous culture step. Furthermore, uncultivable organisms cannot be identified this way. Nevertheless, the use of molecular methods to identify organisms after culture has the potential to be faster than the standard definitive phenotypic identification and antimicrobial susceptibility testing, which require an additional 24–72 h after the culture becomes positive.

Techniques for the identification of pathogens on the basis of their genome can be divided into two categories: hybridization-based and amplification-based. To take advantage of the rapid growth in the availability of automated blood culture instruments, several studies have reported that the use of peptide and/or nucleic acid probes, polymerase chain reaction (PCR) and other nucleic acid amplification techniques can rapidly identify organisms from flagged blood

cultures [41–54]. Despite the advantage of a reduced time to microorganism identification, these techniques cannot reduce the time of blood culture incubation.

3.1. Hybridization techniques for positive blood cultures

Among the techniques suitable for the detection of pathogens in positive blood cultures are hybridization assays with probe matrices and fluorescent in situ hybridization (FISH).

Hybridization probe assays use oligonucleotide probes that are <50 bp long to prevent self-hybridization; the probes have chemiluminescent or fluorescent labels. These complement the sequence of small-subunit rRNA and form a duplex molecule if the target sequence is present. The resulting labelled duplex molecule can be detected. These assays allow the identification of many pathogens within 60 min at the level of genus, species or both, depending on the matrices used [52].

For FISH, slides of growth-positive cultures are prepared on which cells are permeabilized and hybridized with fluorochrome-labelled oligonucleotide probes targeted to rRNA. Fluorescence results from the binding of the oligonucleotide probe to the target RNA, and is visualized by microscopy. FISH allows identification within 2.5 h of more than 95% of the bacteria and yeasts most commonly found in blood [45,55,56]. It is noteworthy that some bacteria are currently identified only at the genus level because no species-specific probes are available [57].

A new FISH method employing peptide nucleic acid (PNA) is commercially available from AdvanDx (Woburn, MA, USA). The first such test, for *Staphylococcus aureus*, uses PNA probes targeting *S. aureus* 16S rRNA to directly identify *S. aureus* from positive blood culture bottles [42,47,53] and distinguishes between *S. aureus* or non-*S. aureus* within hours. This assay has been extended to other bacterial and fungal pathogens [58,59]. These methods can be applied only after Gram stain information is available to drive the choice of the kit type.

Direct identification of the most common Gram-positive bacteria in blood cultures can be performed using a commercially available DNA probe kit (AccuProbe, Gen-Probe Inc., San Diego, CA, USA) that utilizes hybridization protection assay technology [60]. However, the test has been validated by the manufacturer only with the use of freshly grown bacteria from solid media or broth cultures, and not with direct clinical specimens. Lindholm et al. evaluated the diagnostic performance of the test and found excellent identification using a cut-off value recommended by the manufacturer. However, with the *S. aureus* probe, although the specificity was very high (99.8%) the sensitivity was quite low (72.4%). The cut-off values were adjusted and the performance of the tests, especially that for *S. aureus*, was significantly improved [61].

A summary of the principal properties of the commercially available hybridization methods for the identification of pathogens in positive blood cultures is given in Table 2.

[Table 2 here]

3.2. Amplification techniques for positive blood cultures

Amplification techniques can be divided into those that amplify a target, such as PCR, the ligase chain reaction and isothermal amplification techniques (e.g. transcription-mediated amplification and nucleic acid sequence-based amplification) and those based on signal amplification, such as the branched DNA (bDNA) technique. Among the amplification-based techniques, PCR is the most commonly used. Various approaches are available for performing PCR on positive blood cultures: pathogen-specific assays, broad-range assays and multiplex assays.

Pathogen-specific assays are designed to detect one microbial target in the positive blood culture. Their applicability as a diagnostic tool is limited due to the enormous variety of pathogens potentially responsible for BSI.

Broad-range assays are based on the use of primers that recognize conserved sequences of bacterial/fungal chromosomal genes encoding ribosomal DNA. Amplicons must be identified after the PCR through hybridization, sequencing [69,70], polymorphism analysis [71] or subsequent genus- or species- specific real-time PCR [72].

Multiplex assays detect genes of the most frequent pathogens involved in BSI in a single reaction. Amplicons can subsequently be analyzed by electrophoresis

[46], hybridization on an enzyme-linked immunosorbent assay plate [73] or multiplex real-time PCR to identify the pathogen [74].

3.2.1. Broad-range assays

Prove-it Sepsis (Mobidiag, Helsinki, Finland) is an example of a commercially available test based on broad-range PCR performed on DNA extracted from a positive blood culture bottle. The detection of the PCR products is made within the reaction tube, which has a DNA microarray at the bottom. The microarray contains several pathogen-specific sequences, and hybridization of these with the PCR product allows the identification of the specific bacterial pathogen. The results are automatically read by an instrument and interpretation of the findings is made by dedicated software (Prove-it Advisor, Mobidiag). This method is capable of identifying a broad range of pathogens covering more than 90% of the agents usually involved in the aetiology of sepsis. Furthermore, the assay is capable of identifying the presence of the *mecA* gene as an aid to the identification of methicillin resistance in *Staphylococcus aureus* and coagulase-negative staphylococci [75]. Results are available within 3 h and sensitivity and specificity are 94% and 96%, respectively, as declared by the company.

Hyplex BloodScreen (BAG, Lich, Germany) is a multiplex PCR assay with the subsequent identification of several bacterial species by hybridization in an ELISA-like format. The overall turnaround time is approximately 3–4 h and the assay is also available in formats to allow the detection of drug resistance markers, such as *van* genes and several β -lactamase genes [76].

Abbott/Ibis (Abbott Park, IL, USA) recently elaborated a new strategy for the molecular detection of bloodstream infections by coupling broad-range PCR amplification to electrospray ionization/mass spectroscopy (PCR/ESI-MS). This technique, PLEX-ID BAC Spectrum, uses primers designed to target genomic regions highly conserved among bacteria or fungi. Briefly, multiple pairs of primers are used to amplify carefully selected regions of bacterial or fungal genomes; the primer target sites are broadly conserved but the amplified region carries information on the microbe's identity in its nucleotide base composition. Regions like these appear in the DNA that encodes ribosomal RNA and in housekeeping genes that encode essential proteins. Following PCR amplification, a fully automated ESI-MS analysis is performed on the PCR/ESI-MS instrument. The mass spectrometer effectively weighs the PCR amplicons, or mixture of amplicons, with sufficient accuracy that the base composition can be deduced for each amplicon present in the mixture. The base compositions are compared with a database of calculated base compositions derived from the sequences of known organisms. In the event that there is no match for the measured base composition with a sequence in the database, the nearest neighbour organism is identified in a manner similar to that used in the identification of related organisms using sequence data, such as the BLAST search algorithm. The PCR/ESI-MS platform not only identifies organisms present in a clinical sample but is also capable of providing information about the microbe, such as its strain type, whether or not it contains genes that mediate drug resistance and whether or not it carries defined virulence factors [57,77,78].

An important aspect of PCR/ESI-MS is that the method is quantitative [79–81] owing to the presence of an internal calibrant that acts as a PCR control.

3.2.2. Multiplex assays

Several tests based on multiplex PCR have been developed in recent years, and the following three assays are designed to detect only one pathogen and its genetic properties, such as the presence of genes encoding antibiotic resistance.

The StaphPlex system (Qiagen, CA, USA) is designed for simultaneous detection and species-level identification of Panton–Valentine leukocidin (PVL) and several antimicrobial resistance determinants of staphylococci directly from blood culture medium in which Gram-positive cocci in clusters have been seen by Gram staining. The StaphPlex system uses a unique target-enriched multiplex PCR method [82] to amplify and detect 18 *Staphylococcus*-specific genes simultaneously in one reaction: the *tuf* gene target provides identification and differentiation of coagulase-negative staphylococci, the *nuc* gene target is specific for *S. aureus*. The following genes are responsible for drug resistance: *mecA* confers resistance to methicillin, *aacA* is responsible for resistance to aminoglycosides, *ermA* and *ermC* contribute to resistance to macrolides, lincosamides and streptogramins, and *tetM* and *tetK* are responsible for resistance to tetracycline. The amplified products are further characterized by using a Luminex suspension array. The whole process, from processing the blood culture medium to reporting results, can be performed within 5 h, which

greatly shortens the time usually needed for phenotypic identification and antimicrobial susceptibility testing [57,83].

Several pathogen-specific nucleic acid amplification assays are available for differentiating methicillin-susceptible *S. aureus* (MSSA) from methicillin-resistant *S. aureus* (MRSA) and, in some cases, coagulase-negative staphylococci. The first of these to obtain FDA approval in the USA was the StaphSR assay (BD GeneOhm, San Diego, CA, USA). This assay is a multiplex real-time PCR test that is run on the SmartCycler instrument. The assay amplifies specific target sequences of *S. aureus* and a specific target near the *SCCmec* insertion site (*orfX* junction) in MRSA. There are several publications on the performance of the test. While one clinical study [84] performed on 300 blood cultures reported excellent performance characteristics (sensitivity for MSSA and MRSA of 98.9% and 100%, respectively), others noted the limitation of the misidentification of revertant strains [85,86]. Practical considerations regarding this assay are the amount of time required to obtain results (2.5 h) and the expense to the laboratory [20].

There is a single publication on the Xpert MRSA/SA blood culture assay (Cepheid, Sunnyvale, CA, USA) [87]. The primers and probes in this assay detect sequences in the staphylococcal protein A (*spa*) gene, the *SCCmec* inserted into the *S. aureus* chromosomal *attB* insertion site, and the *mecA* gene. In this study, sensitivity and specificity for *S. aureus* detection were 100% and 98.6%, respectively, and for MRSA detection were 98.3% and 99.4%, respectively [87]. Although false positives because of revertant strains in pure

culture are not an issue with this assay, false positives may occur when testing both a methicillin-resistant coagulase-negative *Staphylococcus* sp. and an isolate with a SCC*mec* empty cassette variant in the same sample [87]. The frequency with which this situation occurs varies with geographical location but, in general, is expected to be low. This assay has the advantages of rapid turnaround time (60 min) and random access, but the price per test and initial capital expense are prohibitive for many laboratories. Although not yet cleared by the FDA in the USA, other investigators have developed real-time assays on the LightCycler instrument (Roche, Mannheim, Germany) that target the *mecA* gene and the *S. aureus*-specific *nuc* gene encoding nuclease [88].

Other techniques that involve an amplification step for rapid identification of bacteria in blood culture are the ligase chain reaction (LCR), isothermal transcription-mediated amplification and the bDNA assay. Both LCR and isothermal amplification were first described in 1989 [89,90]. Although these techniques are well known and used for viral load detection in infection with, for example, HIV and hepatitis C virus, they are not used for the detection or identification of bacteria in blood cultures [91–94]. LCR has only been used for the detection of *Mycobacterium tuberculosis* in respiratory specimens, and the isothermal transcription-mediated technique has only been used for the rapid identification of *Candida* spp. in blood culture [95–97]. The bDNA assay is also widely used for the detection of viruses and the measurement of viral load, but its use for bacteria has been described only once, for the detection of the *mecA* gene of *S. aureus* grown in blood [98–100].

A list of amplification tests that can be applied to positive blood cultures is given in Table 3.

[Table 3 here]

Terminal restriction fragment length polymorphism analysis and single-strand conformation polymorphism analysis are mostly used for typing of microorganisms. They have rarely been used for the identification of pathogens grown in blood cultures [71,101].

In summary, molecular identification of pathogens after an initial growth step in blood culture media can be achieved most easily with either hybridization-based or amplification-based techniques. Although initial growth in blood culture is required, most of these assays substantially shorten the time to identification of pathogens or detection of specific resistance genes.

Although molecular identification provides more rapid diagnosis of bacterial infections most commonly found by blood culture, the clinical effect of the shorter turnaround time is still not apparent. At present, the general application of multiplex PCR or broad-range amplification followed by sequence analysis of microorganisms after growth detection in conventional blood culture, and molecular techniques to identify one or few pathogens, does not give much clinical benefit or improved cost-effectiveness compared with conventional identification techniques because they are time consuming and laborious. However, their practicability will likely improve in the near future.

3.3. Mass spectrometry for identifying a pathogen-specific peptide profile

A new approach to the broad post-culture identification of bacterial species has recently been reported, which uses matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF MS) (Bruker Daltonics, Bremen, Germany) [104]. Colonies from an agar plate or liquid culture are mixed with a MALDI matrix and rapidly analysed by mass spectrometry [104,105]. The MALDI-TOF system works by comparing the mass spectral signals obtained from post-culture specimens, on the basis of the organism's proteomic profile, with a database of standard reference spectra. It has also been used to identify bacterial virulence factors [106] and antibiotic resistance markers [107]. A significant advantage of this methodology is that an answer is provided rapidly with a minimal amount of labour compared with conventional methods. Seng and colleagues reported 95.4% success in post-culture bacterial identification by MALDI-TOF MS; 84.1% of pathogens were identified at the species level and 11.3% were identified at the genus level, making this method a candidate to replace Gram staining, which is the most likely niche for this technology in the laboratory [105]. It is still necessary to culture bacteria before analysis by MALDI-TOF, and drug resistance must still be determined by conventional methods. Identifying mixed populations of bacteria will probably be difficult owing to dynamic range issues in the mass spectrometer; La Scola and Raoult reported that of 22 culture samples containing two or more organisms only one species was identified in 18 of the samples [104]. It is likely that the accuracy and

dynamic range of this method will improve over time as the quality of the databases and general methodology improves.

However, due to its ease of use and ability to rapidly speciate, this method has the potential to replace the cumbersome and labour-intensive efforts required to identify the species following culture. Its application in the diagnosis of BSI directly from blood samples is not foreseeable in the near future due to the very low microbial loads observed in most cases. Moreover, the high cost of equipment precludes its routine use in the clinical laboratory, but in the near future it could represent a valid alternative to the biochemical and nucleic acid amplification technology-based identification of cultured bacteria or fungi [57].

3.4. Amplification techniques for whole blood samples

Molecular techniques applied directly on whole blood samples are the best choice for rapid identification of the organism(s) present in blood and, to the extent possible, the genes associated with drug resistance.

A well-designed and clinically validated assay would allow a significantly shorter turnaround time (2–4 days less) than for classical culture-based methods. As for molecular techniques applied to blood culture, several detection strategies have been described, including pathogen-specific, broad-range and multiplex assays. A number of products have received a European CE mark, although none are currently approved by the FDA for use in the USA. Table 4 summarizes the principal properties of whole blood amplification techniques.

[Table 4 here]

3.4.1. Pathogen-specific PCR/genus-specific PCR

These assays, although rarely useful, may be considered when the presence of a fastidious pathogen or slow-growing microorganism is suspected. An example is the detection of DNA from *Streptococcus pneumoniae* in whole blood, because culture-based diagnosis can be difficult. Several studies have shown improved detection of *S. pneumoniae* by PCR in patients with a history suggesting this infection, radiological evidence of pneumonia or treatment with antibiotics and negative blood cultures [108–110]. Although these cases suggest that PCR detection of *S. pneumoniae* in blood is useful, caution is needed because paediatric nasopharyngeal carriage can affect PCR results [33].

An important setting in which pathogen-specific PCR is useful is when rapid diagnosis is needed because life-threatening infection is suspected, such as invasive fungal infections. PCR assays for the specific detection of invasive aspergillosis or candidaemia show good sensitivity (79–100%) [111–113]. The major disadvantage of pathogen-specific PCR assays, however, is that they are applicable only when a specific infection is suspected and they are not useful in the general diagnosis of febrile patients [33].

3.4.2. Broad-range PCR assays

Broad-range PCR assays have been developed for the universal detection of bacteria or fungi in blood. They are based on amplification of the 16S or 23S

rRNA gene of bacteria and the 18S rRNA gene of fungi. Various bacterial species have different numbers of copies of these genes, related to their growth rate. A PCR based on the conserved regions of the 16S or 18S rRNA gene in principle creates the possibility of detecting any bacterial or fungal DNA present in blood. After amplification, the amplicons can be identified by different methods such as capillary sequencing analysis, pyrosequencing or hybridization with specific probes [33].

SepsiTest (Molzym, Bremen, Germany) is a new commercial assay that comprises a combination of whole blood sample 16S and 18S rRNA gene detection for bacteria and fungi, respectively, and sequence analysis of the amplicon for the identification of the microbes. Comparison of this test with blood culture has been performed by Wellinghausen et al., and the results showed that the PCR approach facilitated the detection of bacteraemia in blood samples. This approach potentially allows the detection of any eubacterial or fungal species, but the sequencing approach inevitably extends the turnaround time to 8–12 h, making the clinical usefulness of this test questionable for rapid diagnosis [76].

3.4.3. Multiplex PCR assay

Multiplex PCR involves amplifying multiple targets of DNA in the same sample at the same time using a mix of primers designed to bind the border region to be amplified. This technique is the most promising for routine use for the diagnosis of BSI in clinical microbiology laboratories because it is based on amplification of the internal transcribed spacer. This non-coding region of the ribosomal DNA is

localized among highly conserved genes, shows a high level of heterogeneity among bacterial and fungal genera and species [114–116] and allows a high level of identification using a limited pool of slightly degenerated primers [76].

Among commercially tests the only one available for the detection of microbial DNA in whole blood samples is LightCycler *SeptiFast* (Roche Molecular Systems, Branchburg, NJ). This test is designed to detect the 25 microorganisms that cause approximately 90% of all BSI [117,118]. *SeptiFast* uses real-time PCR in a non-quantitative mode to identify ten bacteria at the species level, several more at the genus level, as well as five *Candida* spp. and *Aspergillus fumigatus*. Fluorescent probes are used, and the melting profiles of the amplicons analysed by dedicated software to identify the pathogens [119].

Clinical studies using *SeptiFast* on blood samples from hospital patient populations with suspected sepsis [120–125], emergency department patients with suspected sepsis [126], febrile neutropenic patients [127,128], neonates [129], or patients with infective endocarditis [130] have been reported. Some important themes have emerged from these studies. Using blood culture as a reference gold standard to compare molecular methods is problematic. As described earlier, culture fails to identify >50% of cases of clinically or otherwise diagnosed sepsis believed to be caused by bacteria or fungi [131].

Unsurprisingly, *SeptiFast* consistently identified more positive specimens than did blood culture methods. These potentially false positives were frequently deemed clinically significant based on review of clinical data [57], other analytical

evidence of infection or disease severity [120] and were often subsequently confirmed after isolation of the pathogen from relevant clinical samples [132]. *SeptiFast*-positive/culture-negative results could conceivably come from non-viable organisms in the blood (resulting from ongoing antibiotic treatment), cell-free DNA released from infected or colonized remote infection sites, or antibiotic interference with culture. Evaluation of the molecular diagnosis of sepsis thus requires a reference method based on multiple data types, as blood culture does not detect many true sepsis cases [131]. On the other hand, culture consistently identified some organisms that were not identified by *SeptiFast*, possibly due to the larger volume of blood analysed by culture and the lower limit of *SeptiFast* detection of approximately 3–30 CFU/ml [119]. In addition, some organisms that cause sepsis are not detectable by *SeptiFast*. Nevertheless, *SeptiFast* and blood culture results were usually in agreement, suggesting that *SeptiFast* can add value as an adjunct to blood culture by both identifying pathogens not identified by blood culture and by identifying pathogens more rapidly than blood culture. *SeptiFast*-negative/culture-negative specimens from patients deemed to be infected based on clinical observations or other molecular markers could result from unculturable organisms, since *SeptiFast* identifies only culturable organisms. The time required to conduct a *SeptiFast* analysis is <6 h; however, the time to the final result in clinical settings may be significantly longer.

These pioneering studies were the first significant commercial attempts to use molecular methods to identify the organisms present in patients with BSI. They

have paved the way for future molecular methods and have established the benchmarks by which the value of newer molecular methods can be assessed.

The LOOXSTER sample preparation system (SIRS-Lab, Jena, Germany) uses a strategy that exploits the methylation differences between bacterial/fungal DNA and human DNA to enrich the clinical sample with pathogen DNA by affinity chromatography. This is followed by 16S rDNA gene amplification using the VYOO test [133]. The amplified products are then run on an agarose gel, with identification being possible through evaluation of the pathogen-specific electrophoretic pattern. As approximately 90% of eukaryotic DNA is removed, signal loss on amplification caused by human DNA is significantly decreased, with sensitivity elevated at least 10-fold compared with samples not subjected to pathogen DNA enrichment. The overall turnaround time is approximately 8 h, and the sensitivity claimed by the manufacturer is 3–10 CFU/ml [76]. Early studies with small numbers of human clinical specimens have shown promising results [122,133].

4. Conclusions

There is a major unmet need to shorten and improve current laboratory procedures for the detection and identification of microorganisms responsible for BSIs. An ideal diagnostic technology would identify the infecting organism(s), and also the determinants of antibiotic resistance, in a timely manner so that appropriate pathogen-driven therapy could begin promptly. Unfortunately, blood culture, the gold standard, largely fails in this purpose; the incubation phase in

bottles and then on solid media is time consuming, and the choice of antibiotic therapy during this period is empirical or driven by the clinical outcome of the patient.

In the same way, the ideal molecular method would analyse a patient's blood sample and provide all the information needed to immediately direct the optimal antimicrobial therapy for bacterial or fungal infections. Furthermore it would provide data to assess the effectiveness of the therapy by measuring the clearance of microbial nucleic acids from the blood over time. None of the currently available molecular method is sufficiently rapid, accurate or informative to achieve this [57].

A realistic, achievable, near-term goal is to analyse blood in parallel with culture methods and identify the pathogens, including unculturable organisms, responsible for infection and some of the key determinants of drug resistance well before the culture results are available. Not all of the molecular determinants of drug resistance are yet known, but some genes have been identified, including those encoding meticillin, vancomycin and carbapenem resistance, that can be measured using molecular methods.

Molecular tests have the potential to be quantitative. Quantitative molecular measurements have been standard in managing chronic viral infections such as HIV and hepatitis C for many years. Several factors preclude quantitative measurements in BSIs. The range of bacterial concentrations in infected patients is broad and the lower end challenges the lower limit of detection of current

molecular methods. Many published studies have shown that the number of bacterial CFU in the blood of adult patients with clinically significant bacteraemia is low, typically in the range of 1–30 CFU/ml [9,134,135]; in children, the levels of bacteria are substantially higher, likely >100 CFU/ml [40]. However, CFU measured by culture represents only the viable organisms that survive the plating process and does not count dead cells, cells that cannot form colonies or free microbial DNA that may have been liberated from lysed cells in the blood compartment. Moreover, infections can be caused by many dozens of different organisms, and quantitative molecular methods that encompass all the potential organisms are lacking. The true concentration of pathogen DNA available for molecular analysis in patients with bloodstream infections is therefore seldom known [57], and several authors support the assertion that the load does not seem to predict the duration of clinical symptoms and does not decline in association with antimicrobial treatment [33].

The implementation of molecular methods must take into account laboratory organization and space. Despite the declared time necessary to perform the test, few laboratories can run sessions on demand and need to batch requests, which introduces a delay. In a study that assumed a one batch per day model, the median time for a *SeptiFast* result was 27 h, whereas it was 18 h for a two batch per day model [121]. The practical considerations of specimen transport, batching specimens for testing and result reporting substantially increase the turnaround time from the theoretical minimum. Nevertheless, even at 27 h after blood collection, the *SeptiFast* results are available more rapidly than blood

culture results, and SeptiFast provides a measurable number of 'gainable days' of adequate antimicrobial therapy [122]. This combination of a shorter time to microbe identification and the ability to detect organisms missed by culture is the value of molecular methods.

A problem with PCR is the increased risk of laboratory contamination in addition to that which might occur when the blood sample is obtained. The main issue is that, as with culture, contamination must always be suspected when unexpected pathogens are identified by broad-range PCR.

What is the clinical interpretation of a positive broad-range or multiplex PCR test?

Molecular methods are technically difficult and merely detect microbial 'DNAemia'. This DNA might originate from pathogens already killed by antibiotics, possibly offering an advantage over culture-dependent systems, but might also represent fragments whose origin is far from clear. The clinical relevance of such fragments to sepsis is far from established. Studies that simultaneously identify the presence of DNA and rRNA, as a measure of viability, could provide useful information about the relevance of detecting DNA in blood. Microbial growth is a cheap, natural PCR and, in our hands, yields high diagnostic performance, admittedly at some time cost. Perhaps the solution is a combination of test technologies to suit individual clinical priorities.

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Table 1

Criteria for defining primary and secondary bloodstream infections (BSI)

BSI

Either isolation of one or more recognized bacterial or fungal pathogens from one or more blood cultures,

or within 24 h of a positive blood culture being collected the patient has at least fever ($>38^{\circ}\text{C}$), chills or rigors; or hypotension (systolic blood pressure <90 mmHg or a reduction of >40 mmHg from baseline in the absence of other causes for hypotension) and there is isolation of a potentially contaminant microorganism from two or more blood samples drawn on separate occasions within a 48 h period or from a single blood sample drawn from a patient with an intravascular line

Primary BSI

Patient has a recognized pathogen (defined as a microorganism not usually regarded as a common skin contaminant, e.g. diphtheroids, *Bacillus* spp., *Propionibacterium* spp., coagulase-negative staphylococci, or micrococci) cultured from one or more blood samples,

or a common skin contaminant (e.g. diphtheroids, *Bacillus* spp., *Propionibacterium* spp., coagulase-negative

staphylococci, or micrococci) cultured from two or more blood samples drawn on separate occasions (including one drawn by venipuncture),

and the organism cultured from blood is not related to an infection at another site, including intravascular devices

Secondary BSI (other than catheter-related)

Patient has a recognized pathogen defined as a microorganism different from a common skin contaminant (e.g. diphtheroids, *Bacillus* spp., *Propionibacterium* spp., coagulase-negative staphylococci, or micrococci) cultured from one or more blood samples,

or the organism cultured from blood is related to an infection with the same microorganism at another site

Table 2

Hybridization techniques for the detection of pathogens in positive blood cultures

Assay	Manufacturer	Principle	Pathogen detected	Turnaround time (h)	Detection limit (CFU/ml)	Refs
PNA-FISH	AdvanDx, Woburn, MA, USA	Fluorescence-based hybridization with peptide nucleic acid probes	<i>Staphylococcus aureus</i> , CoNS, <i>Enterococcus faecalis</i> , other enterococci, <i>Escherichia coli</i> , <i>Pseudomonas aeruginosa</i> , <i>Candida albicans</i> / <i>C. parapsilosis</i> , <i>C. glabrata</i> / <i>C. krusei</i> , <i>C. tropicalis</i>	1.5–3	NA	[58,62–68]
AccuProbe	Gen-Probe Inc., San Diego, CA, USA	Chemiluminescent DNA probes that detect rRNA	<i>S. aureus</i> , <i>Streptococcus pneumoniae</i> , enterococci and group A and B streptococci	2.5	NA	[60,61]

*CoNS, coagulase-negative staphylococci; NA, not available.

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Table 3

Amplification techniques for detection of pathogens in positive blood cultures

Assay	Manufacturer	Principle	Pathogen detected	Turnaround time (h)	Detection limit (CFU/ml)	Refs
Prove-it Sepsis	Mobidiag, Helsinki, Finland	Multiplex PCR combined with microarray	<i>Clostridium perfringens</i> , <i>Enterococcus faecalis</i> , <i>E. faecium</i> , <i>Listeria monocytogenes</i> , <i>Propionibacterium acnes</i> , <i>Staphylococcus aureus</i> , <i>S. epidermidis</i> , CoNS, <i>Streptococcus agalactiae</i> , <i>S. dysgalactiae</i> ss. <i>equisimilis</i> , <i>S. pneumoniae</i> , <i>S. pyogenes</i> , <i>Acinetobacter baumannii</i> , <i>Enterobacter aerogenes</i> , <i>E. cloacae</i> , <i>Escherichia coli</i> , <i>Haemophilus influenzae</i> , <i>Kingella kingae</i> ,	2.5	NA	[75,102, 103]

			<i>Klebsiella oxytoca</i> , <i>K. pneumoniae</i> , <i>Neisseria meningitidis</i> and non- meningitidis, <i>Proteus mirabilis</i> , <i>P. vulgaris</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella enterica</i> ss. <i>enterica</i> , <i>Serratia</i> <i>marcescens</i> , <i>Stenotrophomonas</i> <i>maltophilia</i> , <i>Bacteroides fragilis</i> , <i>Campylobacter jejuni/coli</i> , Enterobacteriaceae,			
Hyplex BloodScreen	BAG, Lich, Germany	Multiplex PCR with subsequent hybridization on an ELISA plate	MSSA, MRSA, <i>S. epidermidis</i> , <i>S. pyogenes</i> , <i>S. pneumoniae</i> , <i>E. faecalis</i> , and <i>E. faecium</i> , <i>E. coli</i> , <i>E. aerogenes</i> , <i>P. aeruginosa</i> , <i>Klebsiella</i> spp.	3	NA	[73]
PLEX-ID BAC	Abbott/Ibis, Abbott Park,	Broad-range PCR combined with	Theoretically hundreds of pathogens	8	NA	[57]

Spectrum	IL, USA	electrospray ionization mass spectrometry				
StaphPlex	Qiagen, CA, USA	Multiplex PCR and characterization of the amplicons by a Luminex suspension array	<i>S. aureus</i>	5	NA	[83]
Staph SR	BD GeneOhm, San Diego, CA, USA	Multiplex real- time PCR assay that amplifies a specific target sequence of <i>S. aureus</i> and a	<i>S. aureus</i> Differentiates MSSA from MRSA	2.5–3	NA	[20,84– 86]

		specific target to detect meticillin resistance				
Xpert MRSA/SA	Cepheid Diagnostics, Sunnyvale, CA, USA	Real-time PCR assay that detects a sequence in the staphylococcal protein A gene and determines meticillin resistance	<i>S. aureus</i> Differentiates MSSA from MRSA	1	NA	[87]

CoNS, coagulase-negative staphylococci; ELISA, enzyme-linked immunosorbent assay; MRSA, meticillin-resistant *Staphylococcus aureus*; MSSA, meticillin-sensitive *Staphylococcus aureus*; PCR, polymerase chain reaction; NA, not available.

Table 4

Commercially available amplification techniques applied to whole blood

Assay	Manufacturer	Principle	Pathogen detected	Turnaround time (h)	Detection limit (CFU/ml)	Refs
SepsiTest	Molzyme, Bremen, Germany	Broad-range PCR followed by sequencing	>300 species	8–12	20–40 for <i>S. aureus</i>	[76]
SeptiFast	Roche Molecular Systems, Branchburg, NJ, USA	Multiplex real-time PCR assay using dual-fluorescence energy transfer (FRET) probes to detect and identify bacterial and fungal	<i>Enterococcus faecalis</i> , <i>E. faecium</i> , <i>Staphylococcus aureus</i> , CoNS, <i>Streptococcus</i> spp., <i>Acinetobacter baumannii</i> , <i>Enterobacter cloacae/aerogenes</i> , <i>Escherichia coli</i> , <i>Klebsiella</i>	6	3–30	[57,117–132]

		pathogens through ITS sequencing	<i>pneumoniae/oxytoca, Proteus mirabilis, Pseudomonas aeruginosa, Serratia marcescens, Stenotrophomonas maltophilia, Candida albicans, C. tropicalis, C. krusei, C. glabrata, C. parapsilosis, Aspergillus fumigatus</i>			
VYOO/ LOOXSTER	SIRS-Lab, Jena, Germany	Multiplex PCR with gel electrophoresis	<i>S. aureus, CoNS, Streptococcus pyogenes, S. pneumoniae, S. agalactiae, several viridans-group streptococci, E. faecalis, E. faecium, Clostridium perfringens, Bacillus cereus, E. coli, E. aerogenes,</i>	8	3–10	[76,122, 133]

E. cloacae, *K. oxytoca*,
K. pneumoniae, *P. mirabilis*,
S. marcescens, *Morganella*
morganii, *P. aeruginosa*,
S. maltophilia, *A. baumannii*,
Burkholderia cepacia,
Haemophilus influenzae,
Neisseria meningitidis,
Bacteroides fragilis, *Prevotella*
buccae, *P. melaninogenica*,
P. intermedia, *C. albicans*,
C. parapsilosis, *C. tropicalis*,
C. glabrata, *C. krusei*,
A. fumigatus

CoNS, coagulase-negative staphylococci; ITS, internal transcribed spacer; PCR, polymerase chain reaction.