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Diagnostic techniques in bloodstream infections: where are we going?

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ABSTRACT

Rapid and early detection of bacteria in blood has an important role in the diagnosis of a febrile patient for at least three reasons: to establish the presence of an infection, to reassure the clinician about the chosen empirical therapy and to define antibiotic treatment after isolation of the microorganism and determination of its antibiotic susceptibility. We all agree that blood culture is the gold standard for aetiological diagnosis. However, it has limitations: the time required for bacteria to multiply to a detectable number of cells, the inadequate sensitivity of blood culture for fastidious pathogens and in patients who have previously received antibiotics, or when there is a catheter-related bloodstream infection. We must, however, remember that the current blood culture data constitute an important epidemiological tool on which clinicians can base empirical therapy. Over the past few years many new molecular tests have been developed that are now entering mainstream practice. These tests are more rapid, specific and sensitive; however, there are still some problems: antibiotic susceptibility testing is still lacking, and sometimes they are so sensitive that a skilled operator may be necessary for an accurate interpretation of the results. These new methods are promising, and their performance can only get better as they are increasingly used in clinical microbiological laboratories.

1. Introduction

Bloodstream infections (BSI) are among the most severe bacterial infections. Despite many achievements in the fields of both microbiological diagnosis and antimicrobial therapy, bacteraemia still accounts for a large proportion of all nosocomial infections – more than 200 000 annually in the USA [1]. A recent European study demonstrated an increase in the incidence of nosocomial bacteraemia caused by multiresistant pathogens [2], and data from a Spanish group demonstrated an incidence of BSI ranging from 16 to 31.2 episodes per 1000 hospital admissions [3]. BSIs have a high mortality, often a prolonged length of stay and increased hospital costs.

The crude mortality rate for nosocomial BSI ranges from 35 to 60% [1,4,5]. The crude mortality in intensive care units is estimated to be approximately 56%, ranging from 31.5 to 82.4% [6,7], and prompt administration of appropriate antimicrobial therapy plays an important role in reducing the mortality associated with this condition [8].

By definition, bacteraemia is the presence of bacteria in the bloodstream. The transient bacteraemia that follows dental manipulation or surgical procedures may have little significance in the otherwise healthy individual with a functioning immune system. By contrast, extensive bacteraemia associated with the release of bacterial toxins into the circulation can be a serious medical emergency leading to bacteraemic shock and eventual vascular collapse. Even transient bacteraemia can be serious for those with prosthetic devices (which can serve as foci for infection) or for those with debilitating medical conditions that increase susceptibility to bacterial invasion.

Detection of bacteria in blood is a key investigation in a febrile patient. It can give important information such as the exclusion of non-infectious causes of the fever, identification of the pathogen, confirmation of the appropriateness or otherwise of the chosen empirical therapy and epidemiological evidence for future empirical therapy. Blood culture has traditionally been the gold standard, and is of great diagnostic value in settings where establishing a microbiological diagnosis is difficult. However, it has limitations: the time to obtain results depends on the time required for a particular bacterium to multiply and attain a significant number of organisms (this time is species dependent [9]), fastidious organisms may fail to grow, and it lacks sensitivity when an antibiotic has been given before blood withdrawal, often despite resin-containing culture fluids.

The latest generation of automated continuous-monitoring blood-culture systems has been a great advance, resulting in earlier detection and better identification of pathogens causing BSI. However, there are many facets of blood culture that are not affected by these improvements and continue to cause problems in the interpretation of results, such as the timing of the culture, the volume and source of the blood, the number of cultures and the type of underlying diseases. Additionally, laboratories should focus on methods of decreasing time to notification, including decreasing the transit time from the patient to the laboratory and prioritizing the processing of blood cultures and Gram-stain results, including a 24 h analytical service. Even addressing all these concerns it will

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be very difficult to improve these systems further without looking at the molecular techniques that are becoming fundamental in the diagnostic microbiology laboratory.

In recent years numerous studies have demonstrated the value of molecular techniques to identify and genotype single bacteria or fungi in 'sterile' specimens. Most of the non-culture-based methods have been developed for the characterization of single pathogens from normally sterile clinical samples. However, in bacteraemia and sepsis a broad range of bacteria and fungi may be present, and this differs in diverse clinical settings, with about 20 bacteria causing 85% of BSI. Much less, but rapidly increasing information is available on these approaches that do not have prior culture steps [10–12].

Nucleic acid-based identification of microorganisms can be used at different times [13]. Firstly, to identify bacteria after growth in conventional blood cultures. In this case all methods used can reduce the time of notification to only 24 h. Secondly, to immediately detect and quantify the DNA in any of the microorganisms present in blood, without the influence of any of the factors described previously in this paper.

2. Molecular methods

Molecular techniques to identify BSI pathogens can be subdivided into four major categories: hybridization-based, PCR-based, microarray methods and protein-based.

The main characteristics of these methodologies are summarized in Table 1. All of these methods aim to identify pathogenic microorganisms within minutes to hours. The interpretation of directly detected pan-bacterial or pan-fungal nucleic acids instead of living microorganisms in blood is complex given the risk of contamination, the ubiquitous presence of bacterial and fungal DNA, and the lack of a gold standard.

2.1. Fluorescence in situ hybridization (FISH)

The best-known hybridization technique used to screen positive blood cultures is fluorescence in situ hybridization (FISH), which can complement conventional culture-based diagnostic methods with differentiation at the species level. The technique is based on the hybridization of fluorescent-labelled probes to a target rRNA, followed by microscopic detection of fluorescence. This method is highly sensitive and specific for detecting most microorganisms in growth-positive blood cultures, with final culture identification within 18 h for bacteria and 42 h for yeasts [10,19]. One study [19] reported that FISH provided identification at the genus level in 91%, and at the species level in 79% of cases; with this method *Staphylococcus aureus* was differentiated from coagulase-negative staphylococci (CoNS) 1.4 h faster than provisional identification. Some other bacteria can be identified only at the genus level; an example is the viridans group of streptococci. As yet, no molecular technique used to identify bacteria in blood cultures provides information on the antimicrobial susceptibility pattern of the organism, which is an important aspect of traditional cultured-based diagnostics. A possible future development could be the transfer of sequence-based identification methods to sequence-based differentiation of antimicrobial resistance. In conclusion, this technique cannot replace standard culture methods, but can be a valuable tool to accelerate the diagnosis in septic patients. The applicability of the method depends on the probes included in the assay, and any attempt to decrease the turnaround time to <1 h would extend the potential use for this methodology.

2.2. PCR-based methods

Among the numerous amplification methods, polymerase chain reaction (PCR) is the most commonly used. Before detailing its possible applications in BSI detection it is very important to make some comments. First, PCR detects DNA rather than living microorganisms; this technique, which reports genes instead of pathogens to clinicians, will have a huge impact on diagnostic procedures in the future. A positive PCR signal in the presence of a negative blood culture can be a real mystery, rendering the results difficult to interpret. Furthermore, to date, PCR-based methods lack good reproducibility; there is a real risk of contamination during the procedure and, no less important, there is no standardization between methods. In spite of these limitations, molecular techniques in diagnostic laboratories are opening a new era. More

Table 1
Molecular techniques for identifying BSI pathogens

Method of detection	Tools and applications	Advantages	Disadvantages	Skills required	References
Blood culture	Gold standard for isolation, identification and determination of susceptibility testing	Detects living organisms Provides antimicrobial susceptibility data	Time-consuming Inhibition by antibiotics Low sensitivity for fastidious organisms	Gold standard	[1,13]
Hybridization-based, after growth in blood culture	Fluorescence in situ hybridization (FISH) with specific probes and microscopic detection of fluorescence	Faster identification of living bacteria	No susceptibility data (yet) Pathogenic specific identification, in some cases only at the genus level Time-consuming	++	[3,10]
PCR-based, after growth or direct detection in blood	Numerous amplification methods, some very promising (i.e. loop-mediated isothermal amplification, PCR and real-time PCR)	Rapid detection and identification No inhibitory influence of antibiotics High sensitivity Quantification of bacterial load possible	No susceptibility data (yet) Risk of laboratory contamination Background bacterial DNA in blood Detection of DNA instead of living organisms	++	[11,14–16]
Microarray	Simultaneous identification of a wide variety of genes	Specific DNA probes spotted on a glass or silicon slide, labelled with a reporter molecule and hybridized to the array Identification and resistance	Low sensitivity of fluorescence-based microarrays Small quantity of DNA Labour-intensive	+++	[11,12,17]
Protein-based	Vibrational spectroscopy (Raman or Fourier transform infrared spectroscopy)	Based on vibrational spectra, which reflect the protein composition of a sample	Value in BSI remains to be determined	++	[18]

experimental data are required to standardize these methods in the clinical context.

PCR-based methodologies can be used for the identification of microorganisms grown in blood cultures; in this case some PCR inhibitors contained in the blood-culture bottles need to be neutralized. PCR-based identification in this case is useful when the bacterial load is low or subculturing is slow, as is the case with mycobacteria, fungi or fastidious organisms. Multiplex PCR protocols can be extremely useful for the rapid identification of *Mycobacterium* spp. in HIV patients [14], but universal identification of bacteria growing in blood cultures in routine diagnosis is less practical because different sets of multiplex PCR assays must be prepared to cover the wide spectrum of pathogens responsible for BSI.

When PCR is used to detect pathogens in the blood we have to be aware that detecting circulating DNA in the blood is not the same as detecting bacteria. Apart from the technical problems related to the use of an amplification method (readers are invited to refer to specific publications for this issue), PCR can provide a sensitive and rapid method for diagnosing bacteraemia in some patients; numerous papers have been published using different treatment protocols for dealing with the sample before amplification (lysis, purification etc.), and amplifying, detecting and reporting the results obtained. Most of these papers describe narrow applications in which a specific pathogen or a specific resistance gene is amplified. In the literature there are data on the sensitivity and specificity of PCR assays performed to identify *Salmonella typhi*, *Mycobacterium tuberculosis*, *M. avium*, *Neisseria meningitidis*, *Streptococcus pneumoniae*, *S. aureus*, the slow-growing HACEK group of organisms, fungi and many resistance genes, including *mec*A [20–25]. Higher levels of *mec*A in blood correlate with higher mortality, indicating the possibility of using this marker as a monitor for MRSA bacteraemia and response to therapy [25].

Universal detection of pathogens in blood is obtained by broad-range PCR assays based on amplification of the 16S or 23S rRNA genes present in all bacteria, or 18S rRNA genes in fungi [11,13]. After amplification the fragments can be sequenced or hybridized with specific probes. A similar result can be obtained by amplifying spacer-specific regions between the 16S and the 23S portions of rRNA. The specificity of this method is increased by the existence of the well known database for this target. In this way, the profile of amplicons obtained can differentiate between many different genera and species.

This sequence is used by the SeptiFast test (Roche Diagnostic, Mannheim, Germany), which uses a multiplex quantitative real-time PCR protocol that, together with a hybridization step, is able to identify species and controls [26]. This technique is based on the measurement of a fluorescent signal generated during each PCR amplification cycle; the intensity of the signal is then related to a standard curve generated by amplification of a known quantity of DNA. This PCR permits calculation of bacterial load. This approach has been successfully applied to the identification of bloodstream pathogens, despite limitations of low bacterial load in adult sepsis and lack of sensitivity for some microorganisms, but its clinical advantage when compared with conventional culture needs to be proven.

2.3. Microarray methods

The microarray is a platform with a wide-ranging potential. Owing to the high level of throughput thanks to miniaturization, microarrays have many applications beyond diagnostic purposes; recent studies have applied microarrays as a research tool towards understanding the aetiology and pathogenicity of dangerous pathogens, as well as in vaccine development. The original emphasis was on DNA microarrays, but the range now includes protein, antibody and carbohydrate microarrays, and research groups have exploited this diversity to further extend microarray applications in the area of biodefence [27].

DNA microarray technology provides the potential for direct and rapid identification of multiple DNA sequences of pathogens responsible for BSI [17]. Briefly, this method consists of DNA probes specific to selected genes, which are spotted on a solid substrate (usually glass) in a lattice pattern. Target DNA to be analyzed is then labelled with a reporter molecule (e.g. a fluorescent dye) and hybridized to the array; specific target-probe duplexes are detected by measuring the fluorescent signals associated with each spot. Major drawbacks of this methodology for pathogen detection are the small quantity of microbial DNA in the analysates and the relatively low sensitivity of fluorescence-based microarrays. This problem was recently addressed in several papers that specifically amplified the pathogen DNA fraction in the sample in order to increase the sensitivity of detection. Random or selective pathogen DNA amplification prior to DNA microarray detection, or a small number of primer pairs corresponding to the capture probes on low-density microarrays were used [28]; recent protocols of large-scale multiplex PCR techniques adapted to the format of a prototype medium-density microarray further increased the detection limit by a factor of 100 to 1000 [12]. It should be noted that the use of these new molecular technologies is not only restricted to detection and identification of microbial pathogens, but can also be used for genotyping, allowing the determination of antibiotic resistance or the performing of microbial fingerprinting.

2.4. Protein-based methods

A completely different approach to the identification of bacteria in blood is that provided by vibrational spectroscopy. No extraction, amplification or labelling steps are needed for this technique. It is based on vibrational spectra, which reflect the protein composition of a sample and can be detected by Raman or Fourier transform infrared spectroscopy. With these techniques, Maquelin and coworkers correctly identified 92% and 98% of pathogens, respectively, in growth-positive blood cultures [18]. Spectroscopy is a new approach to bacterial identification that appears to be promising as a diagnostic technique in the microbiological laboratory. Its value for the detection of BSI remains to be determined.

3. Future perspectives

The use of blood culture to detect pathogens is the gold standard for the specific diagnosis of BSI. As described here, although blood culture maintains some unique characteristics such as combining bacterial identification with antibiotic susceptibility and an unquestioned role in providing epidemiological data, it has many problems related to its relatively long turnaround time and insufficient sensitivity for fastidious organisms or in patients receiving antibiotic treatment. It is very difficult to foresee further improvements from using continuous culture systems, and the development of other options is essential to improve the clinical relevance of this diagnostic tool.

Today we have many promising molecular techniques such as FISH, which, however, require an initial growth step and so cannot be considered a real improvement for patient management.

Detection of bacterial DNA in whole blood with multiplex or broad-range PCR assays is the only methodology able to decrease substantially the turnaround time and not be biased by the inhibitory effect of antibiotics. However, they have a number of limitations which restrict their applicability. The sensitivity of universal PCR is lower than that of many species-specific PCRs, and the contamination of samples and PCR reagents with irrelevant DNA from various sources remains a problem; thus PCR

assays in particular require careful validation for the diagnosis of infection. These problems make the clinical interpretation of results complicated.

The quantitative real-time PCR technique is rapidly replacing the more conventional methods used in the diagnostic laboratory. This technique combines amplification and detection in a closed system, which is faster and reduces the risk of contamination. The possibility of measuring a bacterial load is one of the main characteristics of this system; however, load does not seem to predict duration of clinical symptoms and does not decline in association with antimicrobial treatment [29,30]. Whether this method becomes established in BSI diagnosis remains to be determined. All of these systems lack the possibility of simultaneously identifying antimicrobial susceptibility patterns.

Improving microarray-based techniques could produce a system for identifying a huge number of microorganisms and performing the parallel determination of multiple antibiotic resistance determinants. This could change the management of BSI in the near future. This system has also the potential to perform microbial epidemiology and surveillance at the genetic level; all of these extremely interesting developments could result in a technique with good performance in the clinical microbiology laboratory.

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