

The Era of Molecular and Other Non-Culture-Based Methods in Diagnosis of Sepsis

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INTRODUCTION

Throughout the world, bloodstream infections (BSI) are associated with high rates of morbidity and mortality, with a

mortality rate ranging from 20% to 70% (3, 7, 38–40, 54, 120, 124). Overall, each year, about 750,000 patients develop bacterial or fungal BSI in the United States, resulting in 215,000 deaths (3, 120). Recently, it was estimated that the most dangerous clinical manifestations of BSI, sepsis and septic shock (Table 1), are the 10th leading cause of death in the United States, accounting for 6% of all deaths (50.37 deaths per 100,000 individuals in the overall population) (96). The eco-

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TABLE 1. Definitions for sepsis and organ failure

Syndrome	Definition ^a
Systemic inflammatory response syndrome	≥2 of the following signs and symptoms: WBC count of >12,000 cells/mm ³ or <4,000 cells/mm ³ or >10% immature forms; body temp of >38°C (100.4°F) or <36°C (96.8°F); pulse of >90 beats per min; respiratory rate of >20 breaths per min; or PaCO ₂ of <32 mm Hg
Sepsis	SIRS due to suspected or confirmed infection
Severe sepsis	Sepsis complicated by organ dysfunction, hypoperfusion, or hypotension
Septic shock	Sepsis with hypotension despite adequate fluid resuscitation along with presence of perfusion abnormalities
Multiple-organ-dysfunction syndrome (MODS)	Signs and symptoms of severe multiple-organ dysfunction

^a Definitions reported in this table were derived from data reported in reference 104. WBC, white blood cells.

nomic burden of sepsis-related complications is also very high, with an annual cost of nearly 17 billion dollars in the United States (3). In Europe, an estimated 135,000 patients die each year of sepsis-associated complications, with an overall incidence of sepsis of 3 cases per 1,000 individuals (103). A national prospective multicenter study performed in Germany demonstrated that sepsis is the third most common cause of death in that country, with an overall prevalence of 23.4%, almost equally distributed between severe sepsis (12.4%) and septic shock (11%) (53).

Definitions

The different forms of sepsis are always associated with bacteremia (or fungemia); on the other hand, bacteremia and fungemia do not always cause the syndrome of sepsis. Usually, bacteremia is categorized as transient, intermittent, or continuous (169, 181). The transient form, generally lasting for a few minutes or a few hours, is associated with procedures involving anatomic sites colonized by normal microbial flora (i.e., after colonoscopy, percutaneous catheterization, or dental extractions) or with a manipulation of localized infected sites (i.e., furuncles). Intermittent bacteremia is typically associated with closed-space infections, such as abscesses, or with focal infections, such as pneumonia and osteomyelitis. It is defined as recurrent episodes of bacteremia due to the same microorganism intermittently detected in blood because of cyclical clearance and recurrence of the pathogen at the primary site of infection. Finally, persistent low-grade bacteremia is commonly associated with an intravascular focus of infection such as infective endocarditis (IE) or vascular-graft infections. In all cases the microbial load may be as low as 1 CFU/ml, making the microbiological diagnosis difficult (107, 169, 175, 215).

If bacteremia (or fungemia) is not properly controlled, all of the above-described conditions may be associated with the development of sepsis, a clinical syndrome related to an infectious process with important alterations in the inflammatory response and coagulation. The syndrome of sepsis is a continuum ranging from systemic inflammatory response syndrome (SIRS) to multiple-organ-dysfunction syndrome (MODS). The midpoints are sepsis, severe sepsis, and septic shock (Table 1).

As indicated in Table 1, sepsis is clinically defined when a patient shows at least two of the criteria for SIRS and if there is evidence of systemic infection (15, 104). Sepsis is considered severe when it determines systemic hypotension (systolic blood pressure [SBP] of <90 mm Hg or <2 standard deviations below the normal for age, a mean arterial pressure of <70 mm

Hg, or an SBP decrease of >40 mm Hg) or tissue hypoperfusion and organ dysfunction (15, 104). Septic shock, a complication of severe sepsis, is defined as sepsis-induced hypotension that is not responsive to adequate fluid resuscitation (15, 104).

The Clinical Microbiology Laboratory in the Management of Sepsis

Recently published guidelines for the management of severe sepsis and septic shock include the following key recommendations: (i) prompt administration of broad-spectrum empirical antibiotic therapy within 1 h of diagnosis; (ii) sampling of blood for culture and, if possible, cultures of relevant body sites before the initiation of antibiotic therapy; (iii) prompt resuscitation of the patient within the first 6 h; and (iv) thorough clinical and radiological evaluation of the patient to look for the potential primary source of infection (36). In the acute phase, the role of the clinical microbiology laboratory is usually marginal, as clinicians are aware that at least 24 to 72 h are necessary for the confirmation of an infectious etiology, identification of the pathogen, and evaluation of its antimicrobial susceptibility (11, 21, 156).

The role of blood cultures is crucial for the correct fine-tuning of antibiotic therapy (36). However, several factors such as empirical antibiotic therapy initiated before blood sampling or the presence of fastidious pathogens may have a negative impact on the diagnostic yield of blood cultures even when a bloodstream infection is strongly suspected (48, 51, 65, 78, 79, 98, 131, 136, 153, 204). These technical limitations will not be eliminated by significant improvements in this diagnostic technique. Other approaches are therefore needed, as an adjunct to blood cultures, to improve the overall diagnostic yield.

This review therefore addresses non-culture-based techniques for the diagnosis of sepsis, including molecular methods. Different diagnostic strategies are evaluated, and the potential role of the rapid detection of bacterial and fungal DNA in the development of new diagnostic algorithms is discussed.

CLINICAL ROLE OF BLOOD CULTURES

Blood cultures are the current “gold standard” of BSI diagnosis and are based on the detection of viable microorganisms present in blood. The accurate identification of blood isolates and the identification of their portal of entry are central to the optimal management of BSI. Blood cultures, which are used to detect viable pathogens, have the advantage of allowing the

evaluation of their antimicrobial susceptibility; this characteristic has still not been paralleled by any other technique available to date. This aspect is important, as several studies have shown that inadequate antimicrobial therapy is an independent risk factor for mortality or microbiological failure for severely ill patients with life-threatening infections (81, 100, 102, 105, 206, 217). A recent study of a cohort of patients with septic shock showed that after the onset of hypotension, each hour of delay in the administration of an effective antimicrobial therapy is associated with an average 8% decrease in survival (95).

The Limits of a Gold Standard: Factors Influencing Blood Culture Sensitivity

Current guidelines recommend the collection of two to three blood culture sets per each suspected BSI episode, collecting 20 to 30 ml of blood per each set evenly distributed between an aerobic bottle and an anaerobic bottle (29). Blood cultures are currently performed with continuous-monitoring blood culture systems (CMBCS) using fully automated instruments that incubate the blood samples. These instruments also detect microbial growth by the analysis of CO₂ release using fluorescent sensors (Bactec 9240; Becton Dickinson) or colorimetric sensors (BacT/Alert; bioMerieux, France) or, alternatively, by measuring pressure changes in the bottle headspace due to the consumption and production of gases (VersaTREK; TREK Diagnostic Systems). Several technical developments, including the refinement of culture media and the automated detection of growth, have greatly improved the diagnostic performances of this diagnostic technique (29, 30, 130, 205). However, several factors may still reduce the overall sensitivity of blood cultures.

Blood volume. One of the most important factors influencing blood culture diagnostic yield is blood volume (71, 191). Several studies of adults (4, 17, 30, 84, 126) and pediatric patients (82, 86) confirmed that the rate of isolation from blood cultures increases with the quantity of blood submitted. This is particularly important for pediatric patients, for whom it is not always possible to draw a sufficient volume of blood. A recent study investigating the routine clinical practice at a tertiary children's hospital reported that over half of blood cultures contained an inadequate volume of blood and that these inadequate samples were less likely to yield positive results (2.1% versus 5.2%) (31).

Time from sampling to incubation. Another important variable influencing diagnostic results is the time taken from blood withdrawal to the loading of blood culture bottles into the instrument (177, 180, 199). Ideally, blood cultures should be loaded immediately into the continuous-monitoring instrument in order to minimize the time to detection and to reduce the number of false-negative samples due to delays in loading. An evident decrease in recovery has been observed when bottles are held at room temperature for more than 12 h and even more so when they are preincubated at 37°C before being loaded into the automatic instrument (177, 180).

Fastidious pathogens and antimicrobial therapy. An intrinsic limitation of blood cultures is their low sensitivity to slow-growing and fastidious organisms such as *Bartonella* spp., *Francisella tularensis*, *Mycoplasma* spp., several molds, and *Nocardia* spp. (29, 57). Other uniformly uncultivable pathogens (by the

usual bacterial culture systems) such as *Rickettsia* spp., *Coxiella burnetii*, *Chlamydomphila pneumoniae*, and *Tropheryma whipplei* are better diagnosed by immunodiagnostic or molecular techniques (29, 57). Many of these organisms may be responsible for infective endocarditis, the diagnosis of which is based upon the detection of vegetations on the cardiac valves and positive blood cultures (44). It is not surprising that they are often involved in cases of blood-culture-negative endocarditis (158), confirming the extreme difficulty in microbiologically supporting the clinical diagnosis of IE. Indeed, blood cultures are negative for 2.5% to 31% of suspected IE cases despite the apparent appropriateness of the laboratory procedures (78, 98, 204). The presence of multiple interfering factors such as previous antimicrobial therapy, suboptimal sample collection, or incorrect preanalytical processing may cause false-negative results even in IE cases due to easy-to-culture pathogens such as staphylococci and streptococci (18).

Turnaround time to definitive identification. After a positive signal is given by the automated instrument (usually within 24 to 48 h of incubation [11]), a Gram stain is then performed (together with a preliminary evaluation of the antimicrobial susceptibility by a Kirby-Bauer antibiogram) directly from the blood culture bottle. The pathogen is then identified by biochemical tests. Rapid phenotypic tests may allow the identification of a large percentage of pathogens commonly recovered from blood cultures (usually within 18 to 24 h) (28); however, more time is often needed for the final identification and for antimicrobial susceptibility evaluation of a given isolate, especially when slow-growing pathogens such as yeasts or anaerobes are present (11).

NUCLEIC ACID-BASED DIAGNOSTIC TECHNOLOGIES APPLIED TO SEPSIS

At present, the potential of nucleic acid-based technologies (NAT) has been only partially exploited in the routine microbiology laboratory, especially compared to the molecular biology revolution that has dramatically changed the routine virology laboratory in the last two decades. Indeed, the use of NAT-based diagnostic assays in microbiology has been hampered by the availability of less expensive, less labor-intensive, and often more sensitive culture-based methods. Although this is also true for the diagnosis of sepsis, several NAT-based diagnostic approaches have been described in the literature, and some of them are commercially available. In this section, we review several studies evaluating different extraction methodologies and how they affect the final sensitivity of molecular assays. We then review different NAT-based assays, including those commercially available, while giving a rapid overview of the non-NAT methods applied to positive blood cultures.

Different Strategies and Different Results

NAT-based assays applied to sepsis can be divided into two main categories: NAT assays for the detection and identification of pathogens from blood culture bottles and NAT assays for the detection and identification of microorganisms directly from blood, serum, or plasma samples.

Three types of strategies have been described: pathogen-specific assays targeting species- or genus-specific genes;

broad-range assays targeting conserved sequences in the bacterial or fungal genome, such as the panbacterial 16S, 5S, and 23S rRNA genes or the panfungal 18S, 5.8S, and 28S ribosomal DNAs (rDNAs); and, finally, multiplex assays allowing the parallel detection of species- or genus-specific targets of different pathogens potentially involved in a certain infection type.

Regardless of the strategy used, classical PCR techniques (including nested PCR methods) should be avoided in a high-throughput clinical laboratory, as they are prone to carryover contamination with amplified products (89). PCR techniques based on the automated fluorescence detection of amplicons (real-time PCR) are usually more robust, less labor-intensive, and less prone to contamination than conventional PCR techniques (93, 159). Real-time PCR techniques also allow the absolute or relative quantitation of the target sequence, which can assist in discriminating significant bacterial loads of potentially contaminating species such as viridans group streptococci or coagulase-negative staphylococci (70, 101). However, real-time PCR techniques are certainly more expensive than conventional PCR techniques, and some of the commercially available platforms are still too small, with insufficient throughput. Moreover, the increasing need for full automation will require the combination of real-time PCR instruments with automated robots for the extraction of nucleic acids, leading to higher-throughput workflows but also to a further increase in costs. Not all laboratories will be able to undertake these changes easily, and this will probably contribute to the creation of newer and larger centralized laboratories with highly specialized staff (165). The potential benefits of such a revolution are still open to debate, as reviewed by Raoult *et al.* (165).

Purification of Nucleic Acids from Blood Cultures or from Clinical Samples

Independent of the type of assay used, one of the critical steps in the molecular diagnosis of sepsis, profoundly influencing its sensitivity, is the purification of microbial nucleic acids from blood (184). The accurate purification of DNA from the sample is a prerequisite to any further downstream NAT-based assay. An ideal extraction method should be sensitive, reproducible, cost-effective, fully automated, and universal in its ability to extract bacterial and fungal DNA. Four main drawbacks hinder the diagnostic yield of any molecular assay for the diagnosis of sepsis, and the extraction methodology needs to overcome them: the presence of PCR inhibitors in the blood sample (164), the possible presence of contaminating bacterial or fungal DNA in the reagents (127, 163), the risk of carryover contaminations among samples in the same extraction round (163, 178), and, finally, the interference of high levels of human DNA with the extraction and amplification of less abundant bacterial or fungal DNA (73).

Recent studies compared classical DNA purification methods, including the phenol-chloroform and the alkali wash/heat lysis extraction methods, to commercial kits (114, 127, 128, 163). Many commercial kits are based on spin column technology that can be easily used in a routine laboratory with standard equipment. By using these kits, the nucleic acids are released from cells using chaotropic salts that denature proteins; in some cases, the extraction process is augmented by

digestion with proteinases. The separation of nucleic acids is then obtained by temporary aspecific adsorption onto a glass fiber surface or a silica gel membrane within a disposable plastic column. Subsequent washes with high-salt-concentration buffers then remove denatured proteins and low-molecular-weight compounds; the bound DNA is then purified by the elution of the column with a low-salt-concentration buffer (167).

Nucleic acid extraction from blood culture bottles. A common problem when extracting nucleic acids from blood culture bottles is the presence of PCR inhibitors such as the anticoagulant and anticomplementary agent sodium polyanethanesulfonate (SPS) (62). Millar *et al.* investigated several commercial and in-house extraction methods to isolate bacterial and fungal DNA from BacT/Alert blood culture bottles (128). Most of the methods gave a good yield in terms of recovered DNA, with a higher quantity obtained with the commercial kits. However, all methods but one (the alkali wash/heat lysis method [94]) failed to remove PCR inhibitors present in the bottle broth.

Nucleic acid extraction from whole blood, serum, or plasma. An alternative to DNA isolation from blood cultures is extraction from whole-blood samples or, alternatively, from plasma and serum samples. Extraction from EDTA-treated whole-blood samples provides a higher number of possible target bacteria than methods using serum and plasma, thus potentially improving the overall sensitivity (142). However, the presence of PCR inhibitors in whole blood may make this approach less sensitive (162). As an example, Zerva *et al.* reported a greater sensitivity of serum samples for the diagnosis of human brucellosis by PCR despite the fact that members of the genus *Brucella* are facultative intracellular pathogens (218). More recently, a similar study was undertaken to compare seven commercial DNA purification kits for the recovery of *Brucella melitensis* DNA directly from serum samples, demonstrating that kits containing a proteinase K digestion step yielded the best sensitivity (163). Various rates of cross-contamination between samples have also been described for the different kits, showing that this aspect should also be taken into account when choosing an extraction protocol for a high-throughput routine procedure (163).

Several automated systems for the direct extraction of bacterial and fungal DNA from whole-blood samples have also been developed (91, 108, 109), but the higher costs still limit their use in clinical microbiology laboratories. They are currently used for the high-throughput detection of bacteria or fungi in blood components (187).

Purification of fungal nucleic acids from clinical samples. The purification of DNA from pathogens such as yeasts and molds is certainly more troublesome (197). A significant number of PCR methods have been developed for detecting fungal DNA in either whole-blood or serum samples (91, 179, 211). The low sensitivity of many NAT-based approaches for the detection of fungal DNA appears to be due to the difficulty in breaking the fungal cell wall (110, 114) as well as to the very low loads of fungal cells that may be present in blood during fungemia (in some cases, even less than 1 cell per ml) (110, 212). Indeed, different protocols for the extraction of fungal DNA from clinical specimens have been shown to have a dramatic impact on the overall assay sensitivity (19, 212); as for

bacteria (128), this is related not only to the overall yield of recovered DNA but also to the removal of factors interfering with molecular assays (127).

For a more comprehensive overview of several commercially available extraction kits containing useful information on their salient features, such as detection limit, purity of extracted DNA, processing time, risk of intersample contamination, and cost, we suggest two recent comparative papers by Queipo-Ortuño et al. for intracellular bacteria (163) and by Metwally et al. for fungi (127).

Assays for Identification of Pathogens from Positive Blood Cultures

Several strategies for the detection of bacterial or fungal DNA from blood cultures have been described. These approaches have been applied mainly to positive blood cultures (93, 159) to obtain a more rapid identification of the grown pathogens. Molecular strategies for the detection of fastidious pathogens in negative blood cultures have also been described (178). However, the real diagnostic potential of these approaches is limited, since they do not overcome any of the technical and sensitivity issues sometimes observed with blood cultures; moreover, their clinical usefulness is further limited by the fact that molecular identification may precede the results obtained by culture methods by only a few hours. The role of this faster identification approach may be more relevant when subculturing takes time, as in the case of fungi.

PCR-based methods. Several pathogen-specific, broad-range, and multiplex PCR-based amplification strategies have been used for positive blood cultures.

(i) **Pathogen-specific assays.** The role played by pathogen-specific assays (192) is limited due to the high variety of pathogens potentially responsible for BSI. A partial utility may be recognized for those pathogen-specific assays that are capable of detecting genes encoding resistance to major antimicrobials, such as *mecA* in staphylococci (50, 192) or *van* genes in enterococci (49, 50), thus allowing a 12- to 16-h-shorter turnaround time than the presumptive phenotypic detection of resistance. Overall, the actual clinical impact and the cost-effectiveness of this kind of early notification remain to be proven.

(ii) **Broad-range assays.** The clinical usefulness of broad-range PCR approaches for positive blood cultures is also limited. The main disadvantage of broad-range approaches is that after the PCR amplification of a target sequence, further identification procedures are necessary. Several identification strategies have been associated with the broad-range approach, such as sequencing (85, 161), polymorphism analysis (194), or subsequent genus- or species-specific real-time PCR (64). This approach could be more useful for persistently negative blood cultures in the presence of a strong clinical suspicion of bacteremia and fungemia, as in the case of infective endocarditis (18, 178). The risk of false-positive results due to environmental bacterial or fungal DNA contaminating the extraction kit or the blood culture bottles should always be considered when these panbacterial or panfungal approaches are used (132).

(iii) **Multiplex assays.** A compromise could be achieved by multiplex approaches targeting different genes of the pathogens most frequently isolated from BSI. Several technical solutions have been described, including multiplex PCR with

subsequent analysis of the electrophoretic pattern (111), hybridization on an enzyme-linked immunosorbent assay (ELISA) plate (208), or multiplex real-time PCR (209).

Nonamplified-NAT-based methods. Other molecular methods not based on the amplification of the target have been used for positive blood cultures. An example is fluorescence in situ hybridization (FISH) with oligonucleotide probes targeting bacterial or fungal genes (typically rRNA genes) (87, 207).

An evolution of classical oligonucleotide probes are peptide nucleic acid (PNA) probes, which are synthetic oligomers mimicking the DNA or the RNA structure. In PNA probes, the negatively charged (deoxy)ribose-phosphate nucleic acid backbone is replaced by an uncharged *N*-(2-aminoethyl)-glycine scaffold to which the nucleotide bases are attached via a methylene carbonyl linker (151). Due to their neutral charge, PNA probes have more robust hybridization characteristics than those of DNA probes. As conventional FISH probes, they are usually designed to target naturally abundant rRNA genes, thereby allowing the detection of microorganisms without the need for an amplification step (58, 143, 144, 170). Finally, and adding to their clinical applicability, PNA-FISH probes are less susceptible to inhibition by impurities in different clinical samples than amplified NAT-based methods (20, 59, 90).

Non-NAT-based methods. Non-NAT-based methods used for the direct identification of microorganisms growing in blood culture include conventional immunological and biochemical identification assays performed directly on positive blood culture bottles (26, 35, 63, 166). However, both antigenic and biochemical variations, as well as the presence of more than one microbial species such as in polymicrobial infections, may give rise to a misinterpretation of data, thus requiring the isolation of the pathogen(s) from the blood culture broth.

Among phenotypic assays, new bacteriophage-based pathogen detection assays are also being developed, aiming to improve the efficiency, sensitivity, and rapidity of classical phage plaque assays. These new assays take many forms, including direct visualization of differently labeled phages, reporter phages that genetically deposit trackable signals within their bacterial hosts, and the detection of progeny phages or other uniquely identifiable elements released from infected host cells (6, 171, 172, 176). As far as sepsis is concerned, a phage-based assay (MicroPhage MRSA/MSSA blood culture test; Longmont) for the identification of *Staphylococcus aureus* and the detection of methicillin resistance directly from positive blood cultures is currently undergoing clinical trials in the United States. The assay detects the amplification of *S. aureus*-specific phages in the presence of methicillin with a turnaround time of 5 h. Papers investigating the technical features and the general usefulness of this assay have not yet been published.

Other non-NAT-based techniques (not comprehensively reviewed here) are being investigated for their use in the identification of microbial pathogens directly from blood cultures. The most promising techniques are proteomic technologies, including matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) (121). This technique is able to identify bacteria or fungi by determining their proteomic profiles (195). It has also been used to identify bacterial virulence factors (13) or antibiotic resistance markers (47). This method has the main advantage of allowing a definitive identification, or typing, of isolated microorganisms in

only a few minutes. The main drawback, though, of proteomic techniques is that, until now, they could not be applied directly to biological samples, needing a prior cultivation of bacteria to increase the number of microbial cells for analysis. Their application in the diagnosis of BSI directly from blood samples is not therefore foreseeable in the near future due to the very low microbial loads observed in most cases. Moreover, the high cost of equipment nowadays precludes their routine use in the clinical laboratory, but in the near future, they could represent a valid alternative to the biochemical and NAT-based identification of cultured bacteria or fungi.

Mass spectrometry techniques are also becoming a significant method in DNA analysis and may be used as an adjunct to amplified NAT-based methods for the faster sequencing of amplicons. An example of these hybrid techniques is PCR-mass spectrometry (139, 202). This strategy relies on the PCR amplification and subsequent base composition analysis of the amplified product using MALDI-MS or high-performance electrospray ionization mass spectrometry (ESI-MS). This approach combines the sensitivity of PCR, especially in the broad-range format, with the rapidity of MS techniques, potentially allowing a high-throughput diagnosis directly using clinical samples, including blood (46, 122). Although very promising, more studies of the capabilities of PCR-mass spectrometry techniques to detect microorganisms from different samples are still required to validate their wider use in clinical microbiology.

NAT-Based Assays for Detection and Identification of Pathogens Directly from Blood, Plasma, and Serum Samples

Of more value could be the development and clinical validation of amplified NAT-based assays for the detection and identification of bacterial and fungal pathogens directly from blood, plasma, or serum samples. A well-designed and clinically validated assay would allow a significantly shorter turnaround time (2 to 4 days less) than that for classical culture-based methods. As for molecular techniques applied to blood cultures, several detection strategies have been described, including pathogen-specific, broad-range, and multiplex assays.

Pathogen-specific assays. Several molecular targets have been used for the specific detection of different pathogens (68, 75, 135, 154), but their clinical usefulness in the diagnosis of BSI is limited by the large number of pathogens potentially involved. However, these assays may be taken into consideration when the presence of fastidious or slow-growing microorganisms is suspected, as, for example, in cases of blood culture-negative endocarditis or in cases of possible rickettsiosis, Q fever, bartonellosis, brucellosis, or Whipple's disease (57). In these cases, pathogen-specific assays may be performed on EDTA blood samples, serum, cardiac valves, and vascular biopsy specimens (57). However, the possibility of using other more conventional and less expensive techniques should always be considered. As an example, a *Coxiella burnetii*-specific PCR performed on serum samples has been shown to be useful in the early weeks of infection (before a possible seroconversion), whereas it should not be used later than 4 weeks following onset, when serology has good sensitivity (61).

Genus-specific assays. Genus-specific assays allow the detection of a given group of pathogens without further identification to the species level (56, 92). A condition that could greatly benefit from a genus-specific approach is the detection of invasive aspergillosis (IA). An increasing body of evidence is demonstrating the usefulness of such a genus-specific approach for neutropenic patients with hematological and nonhematological malignancies (92). A recent study documented significantly higher mortality rates (80% versus 35.6%; $P = 0.003$) for patients with probable invasive pulmonary aspergillosis (IPA) according to traditional classification than for patients with IPA who had, in addition, a PCR-based diagnosis by use of bronchoalveolar lavage (BAL) fluid specimens (74). This advantage is probably related to earlier diagnosis with the NAT-based approach (33). A recent meta-analysis evaluating studies using different PCR techniques with blood, serum, or plasma samples and comparing the molecular results with diagnoses made according to European Organization for Research and Treatment of Cancer (EORTC) criteria for IA (5) concluded that a single PCR-negative result is sufficient to exclude possible or probable IA, whereas two positive tests are necessary to confirm the diagnosis (125).

A major problem with molecular assays for the detection of *Aspergillus* spp. is the risk of false-positive results due to contamination by environmental *Aspergillus* spores (92). Indeed, the risk of contamination of reaction buffers or biological samples is high and has to be weighed against the potential diagnostic benefit of PCR testing as a routine procedure (106). Each phase from extraction to amplification has to be strictly controlled. Before extraction, all reagents should be irradiated with UV light, and the extraction should be performed by using semiautomated closed systems, such as the MagNA Pure LC apparatus (Roche Diagnostics, Indianapolis, IN) (32, 108, 109). Alternatively, dedicated hoods should be used for manual extraction. The extraction of a mock sample should also be performed in each session to check for contaminating DNA in the extraction phase. Several extraction instruments also feature an integrated PCR setup apparatus, which automatically dispenses PCR reagents and extracted DNA (32). Alternatively, the use of dedicated PCR setup hoods, preferably equipped with UV light, could help.

Broad-range assays. As far as broad-range approaches are concerned, the combination of universal PCR targeting conserved regions with sequencing (129, 174, 178) or hybridization (196) has also been applied for the direct detection of bacterial and fungal pathogens from blood samples. As discussed above, this approach potentially allows the direct detection of any cultivable or noncultivable bacterial or fungal pathogen. Its application to clinical samples is limited by the same technical factors described above, such as the risk of false-positive samples due to contaminating microbial DNA present in extraction or PCR reagents (132). However, under some conditions, this approach may be useful. An example is the application of broad-range assays for neonatal sepsis, where the performance of blood cultures is often disappointing, with a high number of false-negative samples (168, 178). Some authors argued that for neonatal sepsis, a test with a rapid turnaround time and with a sensitivity superior to that of blood cultures may be desirable even if there are some problems of specificity, such as with broad-range PCR (131). In several culture-negative sam-

ples, Schabereiter-Gurtner *et al.* detected DNA belonging to pathogens such as *Mycoplasma timone*, *Mycoplasma orale*, *Ureaplasma parvum*, *Ureaplasma urealyticum*, and *Treponema pallidum*, which were not detectable with classical approaches (178). On the other hand, other authors reported limited clinical improvement by associating a similar molecular method to classical blood cultures from newborns with suspected sepsis (168). However, the results of that study may be biased by the very low sensitivity of the broad-range 16S PCR used, which detected only 10^3 to 10^4 CFU/ml (the bacterial load in neonatal sepsis is frequently reported to be even less than 10 CFU/ml) (168).

Other broad-range approaches have been used for the microbiological diagnosis of infective endocarditis (18, 118) and for the diagnosis of invasive fungal infections (52, 55).

Multiplex PCR assays and microarrays. Promising approaches for routine use in clinical laboratories are multiplex real-time PCR assays allowing the rapid identification of pathogens directly from a biological sample. In this context, among the most exploited molecular targets are internal transcribed spacer 1 (ITS1) and ITS2 ribosomal DNA sequences. The ITS regions are noncoding sequences interspaced among highly conserved bacterial rRNA genes and fungal ribosomal DNA, showing a high level of heterogeneity among different bacterial and fungal genera and species (37, 117, 138, 160). These characteristics allow the use of a limited pool of slightly degenerated primers for the PCR and species-specific detection with probes targeting the ITS regions (37, 101).

The diagnostic features of both broad-range and multiplex approaches could be exponentially improved, in terms of specificity and the number of identifiable pathogens, with the development of DNA microarrays, that is, microchips coated with a great number of bacterial targets. Some prototype microarrays have already been described and applied to the diagnosis of sepsis (27, 146, 182). One of them also included six antibiotic resistance genes, but it was limited to the detection of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* in positive blood cultures (27). More interestingly, another study compared blood cultures to a 16S rRNA gene PCR microarray assay designed to distinguish 14 genera or species frequently causing sepsis in the pediatric population (182). Among 172 cases of suspected neonatal sepsis, those authors found 8 positive samples by both PCR and blood cultures, whereas 9 more samples were positive only by PCR. Unfortunately, those authors did not report the clinical significance of the samples that tested positive only by the use of the molecular approach, nor did they discuss the clinical usefulness of the earlier diagnosis (182). Overall, the clinical impact of microarrays will be dramatic when a sufficiently broad panel of antibiotic resistance genetic markers (to be adapted according to the geographic distribution of resistance genes) and of virulence markers are available and are applied directly to clinical samples.

NAT-Based Commercial Assays

Molecular techniques to detect fungal and bacterial pathogens in blood can allow a more rapid diagnosis of BSI under several conditions, but their cost-effectiveness in different clinical settings should be evaluated. Moreover, all techniques

have to be carefully controlled from a technical and clinical point of view. Indeed, only validated commercial assays could fulfill the standardization procedures needed, providing consistency among results obtained in different laboratories. Even if wide standardization has not yet been reached, several commercial first-generation molecular assays for the diagnosis of sepsis are already available (Table 2). Most of the reported assays have been approved for diagnostic use in Europe but not in the United States.

Assays for identification of pathogens in positive blood cultures. Among the commercial assays applied for positive blood cultures, the most studied is PNA-FISH (AdvanDx; Woburn, MA) (58, 59, 76, 77, 113, 150, 183, 213). AdvanDx PNA-FISH kits are fluorescence in situ hybridization-based assays, which use fluorescent-labeled peptide nucleic acid probes targeting the rRNA genes of a limited number of bacterial (*S. aureus* or coagulase-negative staphylococci, *Enterococcus faecalis* or other selected enterococci, *E. coli*, or *P. aeruginosa*) and targeting the rDNA of *Candida* species (*Candida albicans*/*C. parapsilosis*, *C. tropicalis*, or *C. glabrata*/*C. krusei*). The different kits are quite easy to use, requiring a hands-on time of approximately 15 min. However, because of the drying phases and the incubation periods, the time to a final test result is approximately 3 h. The reported sensitivities and specificities for the different kits are high, with the mean sensitivity and specificity being very close to 99% and 100%, respectively (58, 76, 150, 183, 213). Pharmacoeconomic studies evaluating the impact of the *S. aureus* PNA-FISH and *C. albicans* PNA-FISH kits are also available. A study of the candida kit demonstrated that a rapid identification of *Candida albicans* in blood cultures allowed substantial cost savings, due principally to caspofungin, which was used empirically in the institution where the study was undertaken (58). On the other hand, the cost-effectiveness of the *S. aureus* kit was challenged by similar results obtained by other much-cheaper tests, such as the classical tube coagulase test performed directly on staphylococcus-positive blood cultures and read after only 4 h of incubation (77).

Another commercial kit used for positive blood cultures is Hplex BloodScreen (BAG, Lich, Germany), a multiplex PCR assay with the subsequent identification of several bacterial species (methicillin-sensitive *S. aureus*, methicillin-resistant *S. aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *E. faecalis*, and *Enterococcus faecium* in the gram-positive panel and *E. coli*, *Enterobacter aerogenes*, *P. aeruginosa*, and *Klebsiella* spp. in the gram-negative panel) by hybridization in an ELISA-like format. The overall turnaround time is approximately 3 to 4 h. A study of 482 positive blood cultures demonstrated a sensitivity for the different species ranging from 96.6 to 100% and a specificity ranging from 92.5% to 100% (208). The assay is also available in formats to allow the detection of other resistance markers, such as *van* genes and several β -lactamase genes.

An interesting new entrant into the market is Prove-it Sepsis (Mobidiag, Helsinki, Finland), the first microarray-based assay designed specifically for the microbiological diagnosis of sepsis. The microarray format allows the detection of a wider panel of bacterial species and of the *mecA* gene. Gram-positive species not detected by the above-described assays but detectable by the Prove-it Sepsis assay are *Listeria monocytogenes*, *Streptococcus agalactiae*, and *Clostridium perfringens*, whereas adjunc-

TABLE 2. Comparison of commercial molecular assays for diagnosis of sepsis reviewed in this paper

Assay	Manufacturer	Salient feature(s)	Detectable pathogens ^a	Detection limit (CFU/ml) ^b	Turnaround time (h)	Reference(s)
Performed on positive blood culture bottles						
PNA-FISH	AdvanDX, Woburn, MA	Fluorescence-based hybridization with PNA probes	Different pathogen-specific kits available	NA	3	58, 59, 76, 77, 113, 150, 183, 213
Hyplex BloodScreen	BAG, Lich, Germany	Multiplex PCR with subsequent hybridization on an ELISA plate	10 different pathogens and <i>mecA</i> gene	NA	3	208
Prove-it Sepsis	Mobidiag, Helsinki, Finland	Multiplex PCR with subsequent hybridization on a microarray	50 different pathogens and <i>mecA</i> gene	NA	3	None
Performed directly on whole blood						
SepsiTest	Molzylm, Bremen, Germany	Broad-range PCR with subsequent sequencing	>300 different pathogens	20–40 for <i>S. aureus</i>	8–12	132
Vyoo	SIRS-Lab, Jena, Germany	Multiplex PCR with subsequent gel electrophoresis	>40 different pathogens and <i>mecA</i> , <i>vanA</i> , <i>vanB</i> , <i>vanC</i> , and <i>bla_{SHV}</i> genes	3–10	8	None
LightCycler SeptiFast Test	Roche Molecular Systems, Branchburg, NJ	Multiplex real-time PCR	25 different pathogens	3–30	6	22, 101, 112, 115, 116, 134, 148, 185, 198, 200, 201, 210

^a See the text for details.

^b NA, not available.

tive gram-negative species that are detectable are *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Campylobacter jejuni*, *Campylobacter coli*, *Bacteroides fragilis*, *Bacteroides vulgatus*, and a wider group of *Enterobacteriaceae*. The turnaround time is 3 h. The company claims 94% sensitivity and 96% specificity, but papers confirming these results are not yet available. Although potentially interesting, the clinical usefulness of this assay is still limited by its use only for positive blood cultures and not directly for blood samples. The improvement of the amplification step may potentially allow its application directly to blood samples.

Assays for detection and identification of pathogens directly on blood samples. Three kits have already been licensed in Europe for the diagnosis of sepsis directly on blood samples: one broad-range and two multiplex PCR assays.

SepsiTest (Molzylm, Bremen, Germany) is a broad-range PCR-based assay targeting the 16S rRNA genes of bacteria and the 18S rDNA of fungi (132). It is characterized by the preanalytic selective degradation of potentially PCR-interfering human DNA in order to improve the overall detection limit of the assay (20 to 40 CFU/ml for *S. aureus*) (132). After PCR, the samples are then run on an agarose gel, and definitive identification is obtained by sequencing. This approach potentially allows the detection of any eubacterial or fungal species, but the postamplification processing of amplicons puts it at a higher risk of false-positive results due to contaminating DNA. Moreover, the sequencing approach inevitably extends the turnaround time to more than 8 to 12 h, making the clinical usefulness of this test questionable for rapid diagnosis.

Vyoo (SIRS-Lab, Jena, Germany) is a multiplex PCR-based

assay addressing approximately 35 bacterial species (*S. aureus*, several coagulase-negative staphylococci, *S. pyogenes*, *S. pneumoniae*, *S. agalactiae*, several viridans group streptococci, *E. faecalis*, *E. faecium*, *C. perfringens*, *Bacillus cereus*, *E. coli*, *E. aerogenes*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Serratia marcescens*, *Morganella morganii*, *P. aeruginosa*, *S. maltophilia*, *A. baumannii*, *Burkholderia cepacia*, *H. influenzae*, *N. meningitidis*, *B. fragilis*, *Prevotella buccae*, *Prevotella melaninogenica*, and *Prevotella intermedia*) and 6 fungal species (*C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, and *Aspergillus fumigatus*). It is also capable of detecting a few genetic markers of antibiotic resistance, such as SHV β -lactamase genes (without distinguishing SHV extended-spectrum β -lactamases), the *mecA* gene, and *vanA* and *vanB* genes. As in the above-described assay, Vyoo also presents an extraction strategy allowing the selective removal of human DNA by a patented affinity chromatography approach using Zn finger motif CpG. The amplified products are then run on an agarose gel, with identification being possible through an evaluation of the pathogen-specific electrophoretic pattern. The overall turnaround time of the assay is approximately 8 h, whereas the sensitivity claimed by the company is 3 to 10 CFU/ml.

Finally, the LightCycler SeptiFast Test (Roche Molecular Systems, Branchburg, NJ) is, to date, the only multiplex real-time PCR assay available for the diagnosis of sepsis. It is capable of detecting genetic material belonging to several bacterial and fungal pathogens, representing approximately 90% of the species responsible for nosocomial bacteremia (101). The LightCycler SeptiFast Test panel includes *S. aureus*, several coagulase-negative staphylococci, *S. pneumoniae*, several

other streptococcal species, *E. faecalis*, *E. faecium*, *E. coli*, *E. aerogenes*, *E. cloacae*, *K. oxytoca*, *K. pneumoniae*, *P. mirabilis*, *S. marcescens*, *P. aeruginosa*, *S. maltophilia*, *A. baumannii*, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, *C. krusei*, and *A. fumigatus*. The assay uses dual fluorescent resonance energy transfer (FRET) probes targeting the species-specific internal transcribed spacer (ITS) regions. DNA is extracted by mechanical lysis with ceramic beads in a Magnalyzer instrument (Roche Molecular Systems), and after purification, it is processed in three parallel multiplex real-time PCRs (gram-positive bacteria, gram-negative bacteria, and fungi). The melting profile of amplified products is then calculated by specific dedicated software, thus allowing the detection of the pathogen and its identification at the genus or species level (101). The detection limit ranges from 3 to 30 CFU/ml, depending on single pathogens, whereas the turnaround time is approximately 6 h (101). The assay has shown promising performances as an adjunct to blood culture for neutropenic (115, 116, 185, 198, 201), pediatric (134, 148, 200), intensive care, and general medicine (112, 210) patients. No real advantage was observed for patients with suspected infective endocarditis (22), but several IE-related bacterial species are not included in the SeptiFast panel, and the sensitivity of the assay may not be sufficient to detect the low-grade bacteremia associated with this condition. In conclusion, the main technical advantage of this assay is its real-time format that markedly reduces the risk of contamination. Current limitations of the assay include its very high cost (€150 to €200 [\$215 to \$290] per test) and the lack of any information on antimicrobial susceptibility.

Principal Shortcomings of NAT-Based Assays for Microbiological Diagnosis of Sepsis

Despite the remarkable technical advances of NAT-based approaches, their widespread use for the microbiological diagnosis of sepsis is still limited by shortcomings influencing their overall cost-effectiveness.

The first point to bear in mind when evaluating molecular reports is that the detection of circulating microbial DNA (DNAemia) does not necessarily indicate the presence of a viable microorganism responsible for a given infection. The high sensitivity needed for the diagnosis of sepsis may increase the risk of false-positive results due to carryover contamination or due to the detection of environmental DNA contaminating the blood sample. Moreover, DNAemia may be the footprint for transient bacteremia not related to any infection (173, 193), or it may be related to the persistence of circulating DNA still detectable several days after successful anti-infectious therapy has been completed (116). More studies are needed to evaluate, for different categories of patients, the clinical usefulness of this new laboratory parameter as an adjunct to blood culture.

Another major drawback of the available molecular assays for the diagnosis of sepsis is that they do not provide information on the antimicrobial susceptibility of the detected pathogen. The rapid detection of a pathogen may allow better fine-tuning of empirical therapy with possible economic savings, but the lack of a specific susceptibility spectrum, especially with multidrug-resistant pathogens on the rise, may limit the clinical

usefulness of these assays. In cases where the presence of a single gene is always associated with phenotypic resistance (i.e., the *mecA* gene for oxacillin resistance and *van* genes for vancomycin resistance), it is relatively simple to design molecular strategies that allow their detection. More troublesome are cases where the phenotypic resistance is influenced by several concurrent factors, such as the regulatory role of distinct genes that modulate the levels of expression of the gene(s) determining resistance.

NONMOLECULAR BIOMARKERS OF SEPSIS: ROLE AND LIMITS

Detection of Fungal Antigens

Fungi continue to be a major cause of infection-related mortality principally for intensive care unit (ICU) patients and patients with hematological malignancies. Frequently, systemic antifungals are necessary for universal prophylaxis and/or on an empirical basis for these high-risk populations, with issues regarding the consequent cost, toxicity, and possible emergence of drug resistance. In addition, epidemiological data suggest that there have been changes in the epidemiology of fungal infections of oncohematological patients (8, 216). Although invasive aspergillosis and candidiasis continue to be the leading fungal diseases, it would appear that less common mold pathogens are emerging, with important therapeutic consequences. Furthermore, it has been highlighted that the introduction of an active diagnostic surveillance strategy for invasive fungal infections of high-risk patients with hematological malignancies may result in improvements in patient survival (9, 25). However, early diagnosis remains a challenge due mainly to the low specificity of clinical symptoms and the low sensitivity of fungal cultures.

GM. The development of an enzyme-linked immunosorbent assay for the detection of galactomannan (GM), an *Aspergillus* sp. cell wall component, has been an important advance for the diagnosis of invasive aspergillosis (155, 188). A positive test confirmed with two sequential samples is considered to be a valid index for invasive aspergillosis diagnosis using EORTC diagnostic criteria (5). Although the detection of GM in serum is easy to perform, a major disadvantage of the serodiagnosis of invasive aspergillosis is the occurrence of false-positive results. Several possible causes of false-positive reactions have been reported, including the intake of certain foods, especially if the intestinal barrier is altered; gastrointestinal chronic graft-versus-host disease; lipoglycans related to *Bifidobacterium* spp.; solutions containing sodium gluconate; and, in particular, the use of semisynthetic β -lactam treatments, especially piperacillin-tazobactam (60). Changes in the manufacturing process for this antibiotic, possibly resulting in the reduction of cross-reacting materials, have been suggested (152). Although the GM assay has been designed to be specific for *Aspergillus* spp., cross-reactivity with other fungal species such as *Blastomyces dermatitidis*, *Penicillium* spp., and *Fusarium* spp. has been reported (34). The increasing clinical impact of non-*Aspergillus* invasive infections warrants caution when interpreting positive GM results, especially if an infection with a potentially cross-reacting species cannot be clinically excluded. Additionally, different studies reported highly variable sensitivities (51, 155).

In a meta-analysis of 27 studies, the overall sensitivity was reported to be 71%, and the specificity was reported to be 89% (155). Even lower sensitivities were reported for high-risk patients, such as recipients of solid-organ transplants (80, 97).

(1→3)-β-D-Glucan. β-Glucan (BG) is another component of the fungal cell wall that present in a wider variety of fungal species, including *Candida* spp. (141). As for GM, different studies reported variable sensitivities (141, 145), with a sensitivity of 69.9% being observed for a large multicenter study using just one sample per patient (145). For most patients with confirmed invasive fungal infections, BG levels were elevated several days before clinical diagnosis (2). It was suggested that BG detection may be a useful adjunctive tool for promptly guiding preemptive antifungal therapy for neutropenic patients (51), but further studies investigating this aspect are certainly needed.

The combined use of GM and BG detection has also been proposed. In a retrospective study of 40 neutropenic patients, parallel detection improved the specificity and positive predictive value of each individual test without affecting the sensitivity and negative predictive values (149). In other words, the combination seems to be useful to identify false-positive reactions for each test. Several studies of animal models seem to confirm this observation (1, 88), but further clinical studies are needed to evaluate the cost-effectiveness of this approach. Finally, it is important that neither the BG nor GM assay detects zygomycetes, a rare but emerging cause of invasive fungal mycosis (24).

Nonmicrobiological, Nonspecific Sentinel Markers in Sepsis Patients

The limitations of blood cultures have also fostered interest in the development of sensitive and rapid laboratory tests aimed at detecting nonspecific biomarkers of sepsis. For this principal reason, assays for C-reactive protein (CRP), procalcitonin (PCT), interleukin-6 (IL-6), and IL-8 and, more recently, proteomic assays have become subjects of interest, and a number of studies have evaluated their clinical usefulness.

IL-6. The release of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-1b, IL-8, and IL-6 in response to infectious pathogens and host injury may lead to SIRS. In particular, IL-6 is induced by TNF-α and can be measured reliably in blood, having a long half-life (147). IL-6, an important mediator in septic shock, has been acknowledged to predict severity and clinical outcome under this condition (69, 119). However, it is relatively nonspecific as a marker of infection, and it was observed that IL-6 levels are elevated under a variety of conditions, such as in the acute-phase response to injury (14, 42, 43), in acute pancreatitis (186), and in renal transplant patients with an increased risk of acute rejection or graft failure (12). From a clinical point of view, IL-6 may be regarded principally as an early predictor of downstream effects, such as organ dysfunction, due to its initial role in the cytokine response.

CRP. CRP is an acute-phase protein synthesized predominantly in hepatocytes and alveolar macrophages (41) in response to a variety of cytokines, including IL-6. CRP also plays a role in immunomodulation. It was observed that CRP modulates the complement cascade and regulates bacterial opso-

nization and phagocytosis (66, 189). Increases in CRP levels have been documented under a variety of noninfectious conditions, including postmyocardial infarction settings (137) and rheumatologic diseases (45). The kinetics of serum CRP are characterized by a rapid response to inflammation and a short half-life (approximately 19 h) (123). Finally, the assay is inexpensive and widely available. Although several studies demonstrated elevated CRP levels in sepsis (23, 157), its nonspecific dynamics cannot support a major diagnostic role for this biomarker in sepsis.

PCT. Procalcitonin (PCT) is a propeptide of calcitonin that is ubiquitously expressed as part of the host's inflammatory response to a variety of insults (10). Although calcitonin is a hormone classically produced in the parathyroids and involved in calcium homeostasis, PCT (one of the calcitonin precursors) has effects on a variety of inflammatory conditions, including cardiogenic shock, trauma, necrotizing pancreatitis, burns, surgery, and infection (10, 203). In animal models of sepsis, an immunological blockade of calcitonin precursors improves organ dysfunction and outcome (140). A growing body of evidence suggests that PCT is a marker of severe bacterial infection (83) and can distinguish patients who have sepsis from patients who have SIRS (133). In particular, PCT levels in plasma have been correlated with sepsis-related organ failure scores and may be useful in risk assessment (123). High and persistent elevations in PCT levels have been associated with poor outcomes for ICU patients (214). Moreover, the value of PCT in risk assessment for pediatric patients was demonstrated (72). Although several studies suggested that PCT is among the most promising biomarkers for sepsis, considerable controversy surrounding its clinical usefulness still remains. A recent meta-analysis (190) indicated that PCT cannot reliably differentiate sepsis from other noninfectious causes of systemic inflammatory response syndrome in critically ill adult patients. However, the U.S. Food and Drug Administration (FDA) has approved the use of PCT in conjunction with other laboratory findings to aid the risk assessment of critically ill patients.

TREM-1. Neutrophils and monocytes/macrophages are the primary mediators of the innate immune response to bacterial infection, promoting the release of proinflammatory cytokines such as TNF-α and IL-1b, which, when produced in excess, contribute to end-organ dysfunction and overwhelming sepsis. The so-called triggering receptor expressed on myeloid cells 1 (TREM-1) is part of the immunoglobulin superfamily and is upregulated in response to bacteria or fungi. When bound to ligand, TREM-1 stimulates the release of cytokines (99). In contrast to infections, TREM-1 is not upregulated in noninfectious inflammatory disorders such as inflammatory bowel disease and SIRS (16). A soluble form of TREM-1 (sTREM-1) is shed from the membranes of activated phagocytic cells and can be quantified in human body fluids. Several studies have investigated the use of TREM-1 as a diagnostic biomarker and have shown it to be more sensitive and specific than CRP and PCT (67). Although TREM-1 may be a promising diagnostic marker for sepsis, less is presently known about its use in risk assessment and prognosis for patients with known sepsis.

Overall, the present data suggest that IL-6 and CRP are sentinel markers of inflammation and infection but are too nonspecific for further clinical use. PCT will likely enhance clinicians' risk assessments for critically ill patients with sepsis.

Furthermore, TREM-1 is an additional promising candidate. Given the high complexity and variability of the disease, biomarker panels or composite markers may prove most useful in examining a particular immunological pathway, predicting organ-specific responses, and, ideally, identifying at-risk individuals.

CONCLUDING REMARKS

The number of people dying from sepsis has almost doubled in the past 20 years, and an increasing number of patients with differing medical conditions are at a high risk of developing sepsis. In this context, a rapid microbiological diagnosis is particularly important for driving the correct therapeutic intervention. The role of surrogate nonmolecular markers of sepsis is still controversial. Although less expensive and more high throughput than molecular methods, their clinical usefulness seems, in the best cases, to be limited in the risk assessment of critically ill patients. Moreover, an increasing body of evidence advocates that the prompt identification of the agent(s) causing sepsis is needed for a more rapid fine-tuning of empirical therapy.

Several molecular strategies have been applied in this field, and a number of technically reliable methodologies have been proposed. These methods may allow the fast and sensitive detection of a wide panel of bacterial and fungal pathogens, also directly from blood specimens. However, these techniques cannot be regarded as substitutes for classical culture methods, since they have been only partially validated for routine clinical use. From this point of view, molecular techniques are only additional assays in the current diagnostic activity for BSI.

An additional point in the evaluation of molecular techniques is that the detection of circulating microbial DNA (DNAemia) represents a new diagnostic parameter that does not necessarily correspond to the presence of viable microorganisms in blood. Although promising, the clinical usefulness of this new parameter needs further validation.

Furthermore, most of the available molecular assays for the diagnosis of sepsis do not provide information on the antimicrobial susceptibility of the pathogen detected. Although the rapid detection of a pathogen normally allows a better use of empirical therapy, the absence of a specific susceptibility profile with multidrug-resistant pathogens may limit the clinical role of molecular methods in sepsis. This point should also be addressed with future studies, especially in cases where the presence of a single gene is not always associated with phenotypic resistance.

Moreover, techniques whose clinical role is mainly to provide sensitivity and rapidity should be available 24 h per day in the diagnostic microbiology laboratory. As a consequence, their availability not only will increase costs and require dedicated personnel but also will modify greatly the organization of laboratories. The cost-effectiveness of such a revolution is still under debate.

In conclusion, the development of a number of first-generation molecular assays for the microbiological diagnosis of sepsis, in support of the use of blood cultures, already opens up a new era in the microbiological laboratory. However, although they offer the hope of potentially better diagnostic assays, they are still not ready for "primetime" use. The refinement of these

assays in the near future (as well as those for other clinical conditions, including central nervous system infections, sexually transmitted diseases, and respiratory infections) will change dramatically the working algorithm and the general organization of clinical microbiology laboratories.

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