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REVIEW

In vitro diagnosis of sepsis: a review

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Abstract: Sepsis, severe sepsis and septic shock, systemic inflammatory response, and other related manifestations represent a relevant medical problem with high morbidity and mortality, despite the improvements in diagnosis, treatment, and preventive measures over the last few decades. The limited knowledge of the pathophysiology in association with the lack of in vitro diagnostic methods for the certain and quick determination of the causative microbiological agents and their antibiotic resistance means the condition is still critical and of high impact in health care. The current gold standard method to detect the sepsis-causing pathogens, which is based on blood culture, is still insufficiently sensitive and slow. The new culture-independent molecular biology-based techniques can lead to the identification of a broad range of microorganisms and resistance markers within a few hours and with high sensitivity and specificity; nevertheless, limitations of, for example, the polymerase chain reaction-based methods still hamper their application in the clinical routine. This review summarizes the in vitro diagnostic methods and their approach in the clinical diagnosis of the bloodstream infections, and explores their advantages and disadvantages at the current state of the art. A quick analysis of the future prospective in multiplex technologies for microbiological diagnosis of sepsis is also provided.

Keywords: PCR, PCR/ESI-MS, microarray, MALDI-TOF, next-generation sequencing, FISH

Introduction

In 1992, sepsis was defined as a systemic inflammatory response syndrome (SIRS) to infection that results from an activation of the innate immune response, regardless of the cause.[1] Sepsis can be associated with acute organ dysfunction, hypoperfusion, or hypotension (severe sepsis) or with arterial hypotension despite adequate fluid resuscitation after attempts of hemodynamic homeostasis maintenance are performed (septic shock).[2] SIRS is considered to be present when there is more than one of the following clinical signs: a) body temperature >38°C or <36°C; b) heart rate >90 beats per minute; c) hyperventilation evidenced by respiratory rate >20/min or $PaCO_2$ <32 mmHg; and d) white blood cell count >12,000 cells/μL or <4,000/cells/μL.[3] In 2003, the International Sepsis Definitions Conference (ACCP/SCCM) clarified that signs of a systemic inflammatory response occur in both inflammatory and non-inflammatory diseases, albeit difficult to analyze, and provided a list to discern sepsis from other conditions. Therefore, the terms sepsis and severe sepsis describe equally infections complicated by organ dysfunctions.[4]

Blood culture (BC) remains the "gold standard" method for pathogens identification in bloodstream infections (BSIs).[5] A significant drawback of this method is that the

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time to positive organism identification may be 12 to >72 hours. Nevertheless, earlier detection of the etiological agent may have the potential to greatly benefit patient care.[6] In the respect of patient's vulnerability, the guidelines for the treatment and management of sepsis recommend the prompt intravenous administration of broad-spectrum antibiotics within 1 hour of diagnosis,[7] considering that a delay in the administration of antibiotic is generally associated with increased mortality.[2,6] Notably, the lack of rapidity and the limited sensitivity of BC have prompted the scientific community to a growing interest in the development of novel molecular assays for detection and identification of BSIs in a much shorter time frame.[2]

The advent of the most recent molecular techniques and technologies is opening a new era in pathogens identification, even if their performance is hampered by the following: insufficient sensitivity; high costs; the presence of, for example, polymerase chain reaction (PCR) inhibitors; and need of highly trained staff.[8] The purpose of the present review is to give an overview on the microbiological tests for the in vitro diagnosis of sepsis.

Epidemiology of sepsis

Sepsis is a clinical syndrome that affects ~2% of patients hospitalized[9] with higher frequency in those admitted to the intensive care unit and the emergency room,[10,11] and with hematological malignancies and following surgery.[12] Age, sex, race, and host genetic factors can influence the incidence and the outcome of severe sepsis, which is higher in infants and elderly than in other age groups, in males than in females, and in blacks than in whites.[10,13] Many polymorphisms in genes encoding cytokines and other mediators of innate immunity, coagulation, and fibrinolysis are involved in sepsis mechanism.[14]

The rate of hospitalizations for sepsis is much higher for those aged ≥65 years (122.2 per 10,000 population) than for those aged <65 years (9.5 per 10,000 population).[15]

Sepsis is one of the leading causes of death in the world with an estimated incidence rate of up to 19 million people worldwide every year.[16] Mortality rates range from 25% to 30% in severe sepsis cases and between 40% and 70% in patients with septic shock.[17]

Recent data declare that incidence rate is increasing each year, due to factors such as aging population, greater use of invasive techniques and surgical procedures, chronic diseases, immunosuppressive drugs, chemotherapy, transplantation, and increasing number of multidrug-resistant microorganisms.[13,18–20]

The central line-associated BSIs in intensive care unit, for example, cause a high mortality rate.[21] In the US, the median rate ranges from 1.8 to 5.2 per 1,000 catheter days, according to the Centers for Disease Control and Prevention.[22]

The most common causes of sepsis are pneumonia and intra-abdominal and urinary tract infections.[23]

Gram-positive organisms are mainly responsible for sepsis,[24] although recently, Gram-negative organisms are isolated in 62% of patients with severe sepsis, while fungi account for 19% of cases.[25]

Streptococcus pneumoniae is one of the most common microorganisms associated to sepsis,[26] more frequent among elderly >64 years,[27] and *Pseudomonas aeruginosa* has the highest mortality (77%) of all.[28]

Pathophysiology

Sepsis, as an immune system-mediated syndrome, is one of the major causes of death, but its pathogenesis is not clear, especially with regard to dysfunction of the immune system.[29]

Systemic inflammatory response is based on a system of cellular activations with release of pro- and anti-inflammatory mediators that contribute to the onset of increasing clinical severity: SIRS > sepsis > severe sepsis > septic shock.[30]

Sepsis develops when the innate immune response becomes amplified and dysregulated, leading to an imbalance between pro- and anti-inflammatory responses with the excessive release of cytokines and other inflammatory regulators.[31]

The early phase of sepsis is characterized by an excessive hyper-inflammatory reaction of the immune system, and subsequently, in the late phase, the release of anti-inflammatory effectors (interleukin [IL]-4, IL-10, IL-13, cortisol, etc) and changes in T cells from Th1 to Th2 can induce a compensatory anti-inflammatory syndrome.[32,33] In this state, with a compromised immune system, the body becomes more susceptible to secondary infections and viral reactivation.[17]

The organ dysfunction in severe sepsis depends directly on cytotoxic effects of inflammatory mediators, bacterial toxins, and tissue hypoxia. Commonly, patients develop the dysfunction of a single organ, which quickly turns into multiple organ failure.[34]

Reactive oxygen species and nitric oxide play a role in the pathophysiology of sepsis, as well.[35] Furthermore, mitochondrial dysfunction has been implicated as a possible causative mechanism for the reduced activity of immune cells in sepsis.[36]

Biological markers of sepsis

The detection of sepsis-specific biomarkers for host response and pathogen identification can lead to both drug development and improved clinical management of sepsis.[37]

Because of the complex pathophysiology of sepsis that involves cell types, tissues, and organ systems, a recent systematic research identified nearly 180–200 distinct molecules as potential biological markers of sepsis. However, none of them has a specificity or sensitivity such that they can be used in clinical practice.[38,39]

Doherty et al defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic process or pathogenic process".[40] Thus, in order to use a biomarker for clinical practice, it must have a high diagnostic accuracy, sensitivity, specificity, and both positive and negative predictive values.[41] Moreover, a combination of several biomarkers may be useful to increase the diagnostic accuracy of the test in terms of sensitivity, specificity, and predictive values.[42]

The biomarkers of sepsis can be classified as markers of acute-phase protein (C-reactive protein [CRP], procalcitonin [PCT], lipopolysaccharide-binding protein), cytokine/chemokine biomarkers (IL-6, IL-8), and markers of other pathophysiologic processes (coagulation factors and soluble cell surface receptors).[43] Currently, complement factors (C3a, C5a, and the soluble form of the C5a receptor, cC5aR) have been defined as early markers of sepsis and sepsis severity.[44] CRP and PCT are the most widely used for the detection of BSIs.[38]

CRP

CRP is a hepatic acute-phase protein[41,45] with plasma concentration of <10 mg/L that markedly increases during the infection (reaching levels >200 mg/L).[46]

This biomarker has been shown to have a higher sensitivity to temperature and white blood cell count, but it is less specific compared to PCT.[47]

Although some authors suggest that higher plasma CRP concentration may help in distinguishing bacterial from viral and other infections, the clinical usefulness of such diagnostic approach remains unclear.[48]

PCT

PCT, a protein of 116 amino acids, is the peptide precursor of calcitonin, involved in calcium homeostasis.[49]

In bacterial infections, it has been demonstrated how plasma levels of PCT start to increase after 4 hours from the beginning of the systemic infection and reach the peak between 8 and 24 hours after.[50]

Plasma PCT levels ≤ 0.5 ng/mL suggest patients are unlikely to be suffering from severe sepsis or septic shock, while levels >2 ng/mL identify patients with high risk.[49,51]

Similar to CRP, it is uncertain if PCT can be used to distinguish between infections caused by Gram-positive and Gram-negative bacteria.[52]

Therapeutic interventions

Sepsis therapy is based on the use of targeted antibiotics that are necessary but not sufficient for the treatment. Guidelines recommend to immediately start a common broad-spectrum therapy (within 1 hour of sepsis diagnosis) in order to cover all likely pathogens.[7] It remains controversial if combination antimicrobial therapy produces a better outcome than adequate single-agent antibiotic therapy in patients with sepsis.[53–55] One major retrospective analysis of sepsis showed an increased mortality of 7.6% for each hour of delay in the administration of appropriate antibiotic.[6]

Despite extensive research in the last few decades to define sepsis and to improve the outcome of patients, it remains a challenge to identify the better therapeutic approach for clinical management and for survival of subjects.

The use of corticosteroids has been widely studied because with their anti-inflammatory properties, they inhibit the production of proinflammatory cytokines.[56] Many studies have not demonstrated beneficial effects in the treatment of septic patients with corticosteroids, while the administration at low doses for a long period can provide benefits in a subset of patients.[57,58]

Early goal-directed resuscitation therapy, a combination of crystalloid infusions, vasopressors or vasodilators, transfusion of packed red blood cells, and dobutamine, is recommended in international guidelines to patients presenting to the emergency department with early septic shock.[59] But currently, its use remains controversial because resuscitation to static measurements of central venous pressure and central venous oxygen saturation did not increase survival of most patients.[60]

One of the effects of SIRS is the reduction of APC, a vitamin K-dependent plasma protein, which has an anti-inflammatory effect; almost all sepsis patients have reduced concentration of APC, and lower levels are associated with worse outcomes. Because of bleeding risk associated with APC treatment, its therapeutic use is indicated only in patients with severe sepsis and organ failure. Administration of Drotrecogin alfa (Activated), a recombinant version of APC, has improved the survival

of patients with severe sepsis and septic shock, who have high risk of mortality.[61]

Increased understanding of sepsis has demonstrated that it is not exclusively an inflammatory syndrome. A delicate balance exists between inflammatory and coagulation systems; in this regard, heparin for its anticoagulant and anti-inflammatory properties might be an ideal, and low-cost, treatment for sepsis.[62] However, animal studies and clinical trials on heparin effectiveness are still limited.

Tissue factor pathway inhibitor has the same properties as heparin, but no study was able to demonstrate the potential benefit; nevertheless, further research is needed to evaluate its role in sepsis treatment.[63]

Molecular techniques for assay of sepsis in positive BCs

BC is the most sensitive method to detect the presence of bacteria or fungi in the blood so that the clinicians can be provided with information relevant for targeted therapy.[64]

BC test consists of laboratory investigations where the blood is inoculated into a proper culture medium and incubated. Media, used in BC bottles, support the growth of the most medically important bacteria and fungi.[65,66]

This test is capable of detecting as low as 1 colony forming unit (CFU) of bacteria or fungi per 10 mL blood.[67]

However, BC value for diagnosing bacteremia and/or sepsis is limited; in fact, up to 50% of all BCs collected are false-positive.[68]

Enriched growth media, adsorbing agents to neutralize growth inhibitors, software for more rapid detection of microorganisms, and growth and advances in automated agitation systems represent recent laboratory improvements for detection of pathogens.[69,70]

BC sensitivity for slow-growing and fastidious organisms can be poor, especially for bacteria that are responsible for community-acquired pneumonia, such as *Mycoplasma pneumoniae*, *Legionella pneumophila*, and *Chlamydia pneumoniae*, and for uncultivable pathogens, such as *Francisella tularensis*, *Bartonella* spp., *Rickettsia* spp., and *Nocardia* spp.[67,71]

A further complication in interpreting a BC test result is the presence of contaminations; in fact, many efforts have been made to reduce contaminated BC through, for example, the use of sterile gloves or prepackaged BC kits.[72]

Moreover, this test, including antimicrobial susceptibility, requires >72 hours for bacteria, and >60 hours for fungi,[69] and BCs are always collected before initiating antibiotic therapy in order to increase the sensitivity.[73]

In spite of these limitations, a positive BC remains an important diagnostic clue or a confirmation for the physicians. The optimal assay result depends on volume of sample, moment of sampling, and accuracy with which this is carried out.[71]

We review different commercially available molecular techniques for the diagnosis of sepsis using positive BC as follows.

Prove-it™ Sepsis

The Prove-it™ assay (Mobidiag, Espoo, Finland) is one of the first commercially available microarray-based assays, which identifies sepsis-causing bacteria and fungi from positive BC in only 3 hours.[74]

The new improved version of Prove-it™ (Prove-it™ StripArray version 2.0) provides more comprehensive answers with less effort. In particular, it detects ~80 species of Gram-negative and Gram-positive bacteria, 13 species of fungi, as well as identifying *mecA*, an antibiotic resistance marker used to identify methicillin-resistant *Staphylococcus aureus*.[75]

The Prove-it™ StripArray is based on broad-range PCR targeting conserved regions of *gyrB*, *ParE*, and *mecA* genes followed by specific microarray that is capable of simultaneously processing from 1 to 96 samples at a time. It consists of eight successive reaction vials with a microchip at the bottom. The assay is easy to process, reducing the risk of human error and providing high throughput and reliability for the routine laboratory diagnosis.[74,76,77]

The main strength of the test is that it offers essential information for the treatment of septic patients 18 hours faster than traditional methods. Several papers have been presented indicating a high sensitivity and specificity (95% and 99%, respectively), although a lower diagnostic sensitivity (62%) has also been observed, nevertheless in a previous study a significantly lower diagnostic sensitivity (62%) was observed.[78–80]

A limitation of this approach is that it does not detect several clinically relevant pathogens, including *Streptococcus viridans*, *Candida* spp., and coagulase-negative staphylococci.[74] Currently, the clinical usefulness of the test is limited because of its use only for BC.

Verigene®

The Verigene® Gram-Positive (BC-GP) and Gram-Negative Blood Culture (BC-GN) Assays (Nanosphere, Chicago, IL, USA) are random-access, automated tests that perform nucleic acid extraction directly from positive BC media, by hybridization to specific oligonucleotide-labeled gold

nanospheres on a microarray. The system consists of a sample processor and a microarray reader. The result can be obtained in ~2.5 hours with <5 minutes of hands-on time.[81,82]

The BC-GP identifies 13 species/genus, including *Staphylococcus* spp., *Streptococcus* spp., *Listeria* spp., and *Enterococcus* spp., and three resistance markers (*mecA*, *vanA*, and *vanB*), whereas the BC-GN detects nine bacteria and six antimicrobial resistance genes (*CTX-M* for the detection of extended-spectrum beta-lactamases and *IMP*, *KPC*, *NDM*, *OXA*, and *VIM* for the detection of carbapenemase).[82–84]

One of the advantages of the assay is that it identifies more rapidly and accurately organisms and their antibiotic resistance genes, compared to traditional methods. For example, a major strength of BC-GP is the ability to differentiate several species of *Staphylococcus*, as well as the *mecA* gene.[82,85] Verigene® is limited to identifying certain species of fungi and Gram-negative bacteria.[83] Another weakness is the lack of published cost-effectiveness analyses.[85]

The BC-GN and BC-GP are reliable, accurate, and rapid assays, which can be integrated into the routine workflow of a microbiology laboratory, even if their clinical benefits should be further evaluated.

These molecular diagnostic technologies enable the clinicians to provide optimized antibiotic therapy more quickly, potentially leading to improved patient outcomes and lower health care costs.

FilmArray®

The FilmArray® (Biofire Diagnostics, Salt Lake City, UT, USA) is a multiplex PCR tool that tests for 24 sepsis-causing organisms and four antibiotic resistance genes such as *mecA*, *vanA/B*, and *Klebsiella pneumoniae carbapenemase* (*blaKPC*).[86,87]

The assay is based on the extraction and purification of nucleic acids from positive BC and amplification of the target genes by a reverse transcriptase first-stage PCR.[88]

This simple system requires just a short hands-on time, with a total run time of ~1 hour, and only one sample can be analyzed at a time.[86,88]

The FilmArray® has a high performance in the identification of both Gram-negative and Gram-positive pathogens; in fact, it presents a sensitivity range from 88% to 100%, and a specificity >98% for all organisms.[86]

In a study reported by Altun et al, the test covered all microorganisms in 91.6% of positive BC bottles, and it had the potential to identify multiple pathogens simultaneously from positive BC with polymicrobial growth.[87]

This approach performs the extraction, amplification, and detection in a closed diagnostic system, minimizing contamination. It is a low-complexity system for the operator, requiring only injection of the BC sample into the pouch and starting the instrument; hence, the laboratory procedures can be performed by personnel with no training in molecular techniques. Another strength is the large number of targets, all evaluated in a single test, covering the majority of organisms determined in both adult and pediatric BC.[88]

Although FilmArray® and Verigene® are reliable and faster, neither of the systems can replace BC.

MALDI-TOF MS

Matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry (TOF MS) (Bruker Daltonics, Billerica, MA, USA; or BioMèrieux, Marcy l'Etoile, France) provides a method for rapid identification of bacteria and fungi (in >90% of BC) by determining their proteomic profiles.[2,89]

This platform functions by ionizing biomolecules (eg, nucleic acids, proteins, saccharides) separated through an electric field according to their mass-to-charge (*m*/*z*) ratio.[90]

Some of the benefits of MALDI-TOF MS, compared to traditional methods, are the rapid time to results (turnaround time of 1–2 hours), the simple protocol for sample preparation (reduced labor load), the low cost, and the ability to directly identify a large range of microorganisms.[91,92] It presents a specificity of ~96% and a sensitivity that varies between 76% and 98% depending on the pathogen.[93]

One of MALDI-TOF MS drawbacks is that it exhibits reduced sensitivity for Gram-positive bacteria and polymicrobial infections with respect to conventional approaches; for example, it is not able to identify different species of the *S. viridans* group.[94] Furthermore, this proteomic technology cannot be applied directly to whole blood; a subculture is necessary.[2]

MALDI-TOF MS, as well as PCR amplification combined to electrospray ionization mass spectrometry (PCR/ESI-MS) (explained in the following paragraph), results in a promising tool in clinical and epidemiological management.

PCR/ESI-MS analysis

A new approach to the diagnosis of sepsis developed by Abbott permits the identification of bacterial species directly from both positive BC and whole blood. The analysis by BC is described later. The test combines a broad-range PCR amplification (it uses primers that recognize bacterial/fungal

conserved genetic sequences encoding ribosomal DNA) with electrospray ionization mass spectrometry (Abbott Molecular, Ibis Biosciences, Carlsbad, CA, USA) for identification of pathogens and associated antibiotic resistance.[95]

Conversely to MALDI-TOF MS, it uses genetic information to identify microorganisms.[96] This permits the ability to detect silent mutations, and to access antibiotic resistance genes, thus providing a mechanism for rapid antibiotic susceptibility testing.

The method uses primers that are designed to target genomic regions sufficiently conserved such that amplification occurs comprehensively within a biothreat cluster. Then, the amplification products are analyzed by mass spectrometry. The turnaround is ~4–6 hours after positive BC,[97] and this assay permits analysis of six samples a time.[98]

This platform shows high analytical accuracy in comparison to routine subculture of BC bottles.[96,99]

A major advantage of this method is the ability to characterize an organism without previous knowledge and to analyze mixtures of microbes directly from raw BC broth without the need for separation of colonies by subculturing.

The platform was initially established on a first model instrument (Abbott Molecular T5000 PCR/ESI-MS). However, the T5000 was hampered by a number of significant mechanical malfunctions; thus, to overcome these limitations, a newer model of this instrument, called the PLEX-ID, has been designed to be more robust and is provided with a software interface more suitable for use in the clinical laboratory.[96,99] The PLEX-ID BAC Spectrum BC assay that runs on the PLEX-ID permits identification of >600 bacteria and *Candida* spp., and it also detects resistance genes for four clinically relevant antibiotic resistance genes *mecA*, *vanA/B*, and *blaKPC*.[97] Further details on the PLEX-ID system for microorganism detection using whole blood are given later in this review.

Hybridization: PNA-FISH® and REBA

Fluorescent in situ hybridization (FISH) tests have been used in rapid diagnosis of BSIs for more than a decade.[100] The technique is based on fluorescently labeled oligonucleotide probes specific for ribosomal RNA, which have been used to detect >95% of bacteria and fungi in BC.[101,102] The most commonly used target in prokaryotes is 16S rRNA.[103] The assay allows the identification of many pathogens in only 2.5–3 hours.[101]

The PNA-FISH (AdvanDx Inc., Woburn, MA, USA) is a new FISH test that uses peptide nucleic acid (PNA) probes, mimicking the DNA or the RNA structure, to detect microorganisms without a step of amplification, thus reducing contamination risk.[104]

Its turnaround is ~3 hours due to the drying phases and the incubation period; the test does not detect as many pathogens as the Prove-it™ assay (only ten microorganisms).[2,105] It presents an excellent sensitivity and specificity >98%.[106]

A comparative study, conducted by Calderaro et al, between the PNA-FISH and the MALDI-TOF MS shows more advantages of the first with respect to the second method. In fact, even if the MALDI-TOF MS is an inexpensive method and a drawback of the PNA-FISH is the limited spectrum of recognized bacteria, the latter requires only basic laboratory equipment, and the results of this study suggest its usefulness in the cases of mixed infections.[107]

The main strength of the assay is the quick procedure that enables a targeted and shorter therapy, increases benefits for patients, and reduces health care costs.[108]

The reverse blot hybridization assay (REBA) is a molecular tool that uses multiple probes for the detection and the simultaneous identification of Gram-positive and Gram-negative pathogens, and *mecA* and *van* genes from BC. Therefore, the REBA test may be able to detect nonviable bacteria or those at a low concentration that are not detectable by BC. Thus, the REBA test may have practical benefits in the clinical setting, particularly for patients on empirical antibiotic treatment before culture results are obtained. Another advantage is that antibiotic resistance can be determined within a few hours of BC sampling.[109]

Molecular techniques for assay of sepsis in whole blood

Techniques for diagnosis of BSI are constantly evolving and need further improvements to reduce the response time in order to have greater potential to positively impact patient care. Molecular assays applied directly on whole blood samples are the best choice to detect the pathogens, although they cannot replace the BC but are complementary to culture-based diagnosis.[110]

LightCycler® SeptiFast assay

The LightCycler® SeptiFast assay, a multi-pathogen probe-based real-time PCR (Roche Diagnostics, Basel, Switzerland), detects and identifies a wide range of bacteria and fungi causing BSIs directly from 1.5 mL whole blood, without pre-culture.[111]

The test is based on dual-labeled fluorescent energy transfer probes targeting the species-specific internal transcribed spacer regions of bacteria and fungi.[77]

The LightCycler® SeptiFast assay is capable of identifying 25 different sepsis-causing pathogens and the *mecA* gene associated with methicillin resistance in *S. aureus* in <6 hours. The detection of the pathogen by this molecular diagnostic tool permits the rapid diagnosis of bacteremia/fungemia and an earlier administration of appropriate antibiotic therapy.[112,113]

The detection limit is 100 CFU/mL for *Candida glabrata*, *Streptococcus* spp., and coagulase-negative *Staphylococcus*, and ranges from 3 to 30 for the others, depending on the infective agent.[114]

Limitations include its high cost (150–200 € per test), trained personnel requirement, and lack of information on antibiotic sensitivity.[2,115]

The assay has shown variable levels of sensitivity and specificity;[111,116] in particular, sensitivity was 80% and specificity 95% for bacteria, whereas 61% and 95% for fungi.[79,114]

Several studies on neutropenic, general medicine, and intensive care patients have shown reliable results in the diagnosis of BSIs through a combination of SeptiFast and BC assays, particularly when a fungal infection is suspected.[117–119]

Furthermore, other literature data suggest that SeptiFast might be a valuable complementary tool to manage patients with clinically suspected sepsis.[120]

This assay represents an important alternative to BC, mostly for its short time to get the result and the high specificity, although further advancements in laboratory staffing and workflows are necessary to improve its suboptimal sensitivity.

SepsiTest®

The SepsiTest® (Molzym, Bremen, Germany) includes DNA extraction of samples and PCR or real-time PCR using primers targeting conserved regions of the 16S and 18S rRNA genes of bacteria and fungi. After PCR, gel electrophoresis and sequencing are performed. Results are considered positive if sequencing is successful.[121]

A broad spectrum of Gram-negative, Gram-positive, and fungal organisms (>300) were identified by this assay. Compared to BC, this approach displays a diagnostic sensitivity and a specificity of 87% and 86%, respectively.[122]

One of the advantages of this procedure is that low blood volume is required.[123] Conversely, the procedure is limited by its turnaround (requiring from 8 to 12 hours of work) due to several steps (DNA extraction, PCR amplification, sequencing, online sequence identification), and consequently, the risk of contaminations is increased.[124,125]

In a multicenter study that evaluates the SepsiTest® diagnostic accuracy, the assay was applied on 342 samples from 187 patients with SIRS, sepsis, or neutropenic fever; the results showed a higher rate of positivity compared to BCs.[122]

As soon as its cost and time to result decrease, the assay will be considered a reliable tool for clinicians in patient care and will represent a useful supplemental method in the diagnosis of sepsis.

VYOO®

The VYOO® (SIRS Lab, Jena, Germany) is a diagnostic multiplex PCR method for qualitative detection of specific bacteria and fungi from 1.0 to 5 mL blood sample. It consists of mechanical lysis of whole blood, automated total DNA extraction, and pathogen DNA enrichment by affinity chromatography. Then, the pathogen-enriched DNA is amplified and analyzed by a run on agarose gel.[77,79]

The test specifically identifies 39 sepsis-related pathogens (32 bacterial species and seven fungal species) and five resistance genes such as the *mecA*, *vanA/B*, *B-lactamase blaSHC*, and *blaCTX-M* genes.[2,77,79]

The main advantage of this PCR-based assay, compared to BC, is the turnaround (8 hours vs 1–3 days),[70,79] while the limitation is the number of pathogens that may be identified at the species level and the low specificity of the electrophoresis-based identification.[110]

The VYOO® assay has been used in a study in which 24 patients with severe sepsis and 22 with SIRS were examined. In sepsis patients, the VYOO® gave more positive results than BC, whereas in SIRS patients, BC was positive in five cases, but no pathogens were determined by the PCR.[126]

Using the VYOO® assay, clinicians can rapidly and reliably identify causative pathogens as well as important antibiotic resistance markers. A main limitation for its clinical validation is the technical training required to support the laboratory procedures.

Magicplex™ sepsis real-time

Magicplex™ assay (SeeGene, Seoul, Korea) is a multistep approach that associates conventional PCR and real-time PCR and screens for >90 pathogens (73 Gram-positive, 12 Gram-negative, and six fungi) as well as three drug resistance markers (*mecA* and *vanA/B*) from whole blood samples. Also, 27 pathogens can be identified to the species level.[124] The method uses the SelectNA™ blood pathogen kit to extract DNA, requiring <1 mL of whole blood, while a dual priming oligonucleotide (two functional priming

regions separated by a polylinker) is used for the amplification step.[127]

It provides results within 6 hours including several steps (pretreatment of specimens, extraction of DNA, screening amplification, and amplification to identify the pathogens) and different devices.[124,127]

In the first study that has evaluated the clinical performance of the assay,[127] Carrara et al have shown a nearly equal sensitivity compared to BC (65% vs 71%). The sensitivity value was comparable to the rate measured in another study (64%), as well as the specificity value (92% vs 96%).[80] Conversely, Loonen et al while investigating the performance of the assay found a sensitivity and specificity of 37% and 77%, respectively.[124] Nevertheless, despite its low sensitivity, the test is an accurate tool to determine bacteria, mostly those belonging to *Enterobacteriaceae*.[128]

A negative point of the Magicplex™ is the contamination risk of PCR reagents by amplicons.[127]

Further advancements in the technology (eg, for processing a larger volume of blood to increase diagnostic sensitivity) are necessary to improve its clinical validation.

PLEX-ID

PLEX-ID (Abbott Molecular, Ibis Biosciences, Carlsbad, CA, USA) is a novel and universal method for diagnosis of a broad range of pathogens and four resistance markers (*mecA*, *vanA/B*, and *blaKPC*) directly from the patient's blood.[129]

As previously described, this process includes automated DNA extraction, PCR setup, PCR amplification, amplicon purification, and PCR/ESI-MS. It consists of PCR, using nine primer pairs targeting 16S rDNA, 23S rDNA, and four housekeeping genes, and ESI-MS for amplicon analysis.[95,97]

In a newer version of PCR/ESI-MS, IRIDICA (Ibis Biosciences), the volume of blood used is enhanced (5 mL instead of 1.25 mL) in order to increase sensitivity. Furthermore, improved sensitivity is also achieved by the use of specific primers.[129]

Another improvement is that one to six specimens can be analyzed at a time.[97]

Rapid turnaround time (5–6 hours) in both is provided by the use of ESI-MS rather than Sanger sequencing.[130]

This approach shows a sensitivity of 83% and a specificity of 94% compared to BC.[131] A major strength of PLEX-ID is the possibility to evaluate both sterile and nonsterile blood samples. IRIDICA is able to detect an extra 80 microorganisms that did not grow in BC.[110,129]

Nowadays, the IRIDICA technology represents a reliable test in the diagnosis of BSIs directly from whole blood, but more studies on costs/benefits are needed to evaluate its use in the clinical diagnostics.

Tables 1 and 2 summarize the main characteristics of assays for identification of microbes directly from BC and whole blood.

Table 1 Commercially available molecular techniques for the diagnosis of sepsis using positive blood cultures

Assay	Manufacturer	Principles of the assay	Pathogens detected (N)	Execution time (hours)	Sensitivity (%)	Specificity (%)	Ref
Prove-it™ Sepsis	Mobidiag, Espoo, Finland	Multiplex PCR combined with microarray	83 pathogens (19 GP, 51 GN, 13 fungi), 3 DRM	3	94.7	98.8	74
Verigene® BC-GP	Nanosphere, Chicago, IL, USA	Hybridization on microarray	9 species, 4 genera, 3 DRM	2.5	50–100	98.8–100	81,83,84
Verigene® BC-GN	Nanosphere, Chicago, IL, USA	Hybridization on microarray	5 species, 4 genera, 6 DRM	2	50–100	99.4–100	83,84,132
FilmArray®	Biofire Diagnostics, Salt Lake City, UT, USA	Multiplex PCR system	24 pathogens (8 GP, 11 GN, 5 species of *Candida*), 4 DRM	1	88–100	>98	79,86,87
PNA-FISH	AdvanDx Inc., Woburn, MA, USA	Fluorescence in situ hybridization with PNA probes	12 pathogens, (5 GP, 2 GN, 5 species of *Candida*)	2–3	99	100	2,70,134,135
MALDI-TOF MS	Bruker Daltonics, Billerica, MA, USA; or BioMèrieux, l'Etoile, France	Mass spectrometry	Hundreds of pathogens	1–2	76–98	>96	91,93,133
PLEX-ID BAC	Abbott Molecular, Ibis Biosciences, Carlsbad, CA, USA	Multiplex real-time PCR/ESI-MS	>600 bacteria and *Candida* species of 4 DRM	4–6	95	98.9	96,97,99

Abbreviations: PCR, polymerase chain reaction; GP, Gram-positive; GN, Gram-negative; DRM, drug-resistant marker; BC-GP, Gram-positive blood culture; BC-GN, Gram-negative blood culture; PNA-FISH, peptide nucleic acid-fluorescent in situ hybridization; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; Ref, reference.

Table 2 Commercially available molecular techniques for assay of sepsis in whole blood

Assay	Manufacturer	Principles of the assay	Pathogens detected (N)	Execution time (hours)	Sensitivity (%)	Specificity (%)	Ref
LightCycler®SeptiFast Test MGRADE	Roche Diagnostics, Basel, Switzerland	Multiplex real-time PCR	25 pathogens (10 GN, 9 GP, 6 fungi), *mecA* genes	6	79.0–91.3	88.1–96.0	79,111,114,116
SepsiTest®	Molzym, Bremen, Germany	Broad-range PCR with sequencing	>345 pathogens	8–12	87.0	85.8	2,77,110,122
VYOO®	SIRS Lab, Jena, Germany	Multiplex real-time PCR with electrophoresis	39 pathogens (14 GP, 18 GN, 7 fungi), 5 DRM	8	60.0	75.0	2,77,79,110,136
MagicPlex™ sepsis real-time	Seegene, Seoul, Korea	Multiplex real-time PCR	91 pathogens (73 GP, 12 GN, 6 fungi), 3 DRM	6	65	92	79,127,135
IRDICA	Abbott Molecular, Ibis Biosciences, Carlsbad, CA, USA	Multiplex real-time PCR/ESI-MS	Up to 800 pathogens, 4 DRM	6	83	94	95,96,129,131

Abbreviations: PCR, polymerase chain reaction; GN, Gram-negative; GP, Gram-positive; DRM, drug-resistant marker; ESI-MS, electrospray ionization mass spectrometry; Ref, reference.

Sequencing

MicroSEQ 500 Kit (Perkin-Elmer Applied Biosystems, Waltham, MA, USA) and pyrosequencing (Biotage, Uppsala, Sweden) represent sequencing technologies to identify microorganisms in positive BC and from whole blood in a fast turnaround and with lower costs compared to conventional Sanger method.[70,137] The first involves amplification and sequencing of the first 527 bp fragment of the 16S rRNA genes of bacterial strains,[138] and the second has been used to classify and identify a variety of bacterial 16S rDNA fragments.[137]

Currently, the next-generation sequencing (NGS) technology (eg, Illumina MiSeq) represents a new challenge to identify and genotype viable, dead, and viable but nonculturable pathogens, and antibiotic resistance markers, and it provides significant input to scientific discovery due to its cheap cost and fast turnaround.[92,139,140]

The biggest challenge in applying NGS to BSIs is the detection of very small amounts of pathogen nucleic acid in the vast excess of human genomic DNA.[79]

Discussion and conclusion

The high incidence and severity of systemic infections can be ascribed to several factors, among which are the immaturity of the immune defense mechanisms and the complex interactions between pathogens and host.

Sepsis, in fact, is a pathological condition triggered by microorganisms and induced by inflammatory mediators, which causes alteration of the immune, inflammatory, and coagulative equilibrium. Disease progression and clinical manifestation depend on a complex and delicate balance between pro- and anti-inflammatory factors.[31]

The clinical evolution of disease can greatly differ among subjects and sometimes be extremely fast and severe; therefore, an early and rapid identification of the causative agents and the sequential application of the targeted therapies are the necessary conditions for improving patient survival. Strategies have thus to be started on the primary care level in order to manage all patients outside of modern intensive care.

The incidence of sepsis continues to increase, and its diagnosis remains a major challenge for immunosuppressed subjects, given that over half of patients suspected of having sepsis are culture-negative.[141]

Incorrect diagnosis and inappropriate antibiotic administration seem to be the most common causes of sepsis-associated mortality. It has thus become an increasingly urgent need to search for new methods to improve sensitivity and speed of diagnosis.

BC is still considered the "gold standard" for treatment of sepsis, even if in many cases it remains a tool insufficiently sensitive and too slow.[9]

The response time of BC can be 2 days or longer; furthermore, in particular for certain disorders, it is too long to allow physicians to make a targeted antibiotic therapy. Despite recent progress to optimize the technique, the sensitivity for the detection of certain pathogens remains poor, and the contamination of BC continues to be a significant problem. Community-acquired pneumonia, invasive fungal infections, and endocarditis are caused by pathogens difficult to detect, that therefore are responsible for high mortality in untreated patients. The contamination rate is still estimated to be ~3%, despite operators with specialized expertise being involved in sampling and laboratory procedures, and that prepackaged BC kits and sterile material have been introduced to reduce the contamination.

Several studies have shown that many factors may reduce the sensitivity of BC. The most important are the blood volume and the time from sampling to incubation. The first is confirmed

in studies on pediatric patients where the rate of isolation from BC increases with a low quantity of blood submitted,[142] whereas the second considers that BC should be processed immediately to decrease the number of false-negative samples.[143]

Recently, new methods have been developed to reduce the time of diagnosis, and to improve the sensibility and the clinical benefits of detection of pathogens. The molecular detection techniques have allowed early identification of more pathogens and important resistance genes compared to conventional BC.

The short turnaround times of molecular assays may be of clinical importance in the management and the outcome of sepsis patients, since a delayed antibiotic treatment significantly increases mortality rates. In this respect, some research shows that culture-independent molecular assays, such as LightCycler® SeptiFast (Roche Diagnostics) and Magicplex™ assay (SeeGene), are very swift with a turnaround time between 3.5 and 6 hours.[114,124]

Moreover, the opportunity of reducing the time to set up an optimal and effective antimicrobial therapy has allowed a drastic reduction of health care costs associated with inappropriate treatments resulting in prolonged length of hospital stay.

BC permits detection of very low numbers of infecting agents,[144] while the molecular assays directly from whole blood cover a broad range of pathogens causing sepsis.[145,146] In this respect, literature data document that SepsiTest® (Molzym) and PLEX-ID (Abbott Molecular) identify the largest number of pathogens (>300[122] and up to 800 microorganisms, respectively).[95]

A limit of PCR-based assays is the impossibility to provide information on antimicrobial susceptibility of the detected pathogen.

Another drawback of these tools is their high cost that includes need of equipment, reagents, and skilled personnel available for a long time.

MALDI-TOF MS and PCR/ESI-MS, due to their rapid pathogen detection, reduced labor load (specialized personnel is not required), and inexpensive methods, are widely used in the identification of microorganisms in BC and directly from whole blood; nevertheless, their performance is hampered by their limited ability to test for antimicrobial susceptibility of bacteria.

Hence, despite the advantages of molecular techniques in terms of sensitivity and promptness, the antibiotic resistance spectrum can only be achieved by BC. Thus, none of the molecular tests can replace BC, but they are complementary and must be applied in combination in order to reach a correct and faster diagnosis.

Further advances in the near future are required in order to overcome the shortcomings of BC- and PCR-based assays. The new techniques should improve the limited analytical sensitivity for determination of pathogens difficult to detect and to distinguish between viable and dead bacteria.

A first step in this regard is the development of the Genalysis® platform produced by DNA Electronics Ltd (London, UK),[147] which can perform both PCR and sequencing on the same chip in the same analysis to arrive at a fast, accurate, and informative diagnosis.

NGS technologies allow a fast identification of pathogens (2–3 hours) and have the potential to reveal at the same time the pathogen specimens and antimicrobial susceptibility.

Future research and technological developments should be performed to find bioinformatic tools and sequencing platforms that speed up the procedure, and become simpler and less expensive in order to get to the "new gold standard" for BSI diagnosis.

Disclosure

The authors report no conflicts of interest in this work.

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