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NON-CULTURE-BASED METHODS TO DIAGNOSE BLOODSTREAM INFECTION: DOES IT WORK?

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Bloodstream infections are a major cause of morbidity and mortality worldwide. Molecular methods for the detection of pathogens in blood have been developed. The clinical utility of these methods and their integration into the clinical workflow is discussed.

Keywords: bloodstream infection, sepsis, septic shock, blood culture, SeptiFast, SepsiTest, Vyoo, molecular methods

Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) that is caused by an infectious process, and severe sepsis and septic shock represent more severe levels of sepsis [1]. In a subgroup of patients with sepsis, bacterial or fungal pathogens are circulating in the blood, which is called bloodstream infection. Sepsis, severe sepsis, and septic shock cause significant morbidity and mortality among populations worldwide [2]. The diagnosis of the underlying cause of sepsis is a considerable challenge to physicians in intensive care units, ordinary wards, and emergency care. Since mortality in patients with septic shock is dependent on early initiation of antimicrobial therapy, tools for a rapid and accurate diagnosis are desired [3]. Current methodology relies mainly on conventional microbiological techniques: the culture of microorganism in enriched broth, isolation of the pathogen, identification through biochemical properties, and susceptibility testing. With the aim to increase the speed of diagnosis, molecular techniques have been developed.

Blood culture

In a bloodstream infection, viable microorganisms are present in blood. The density in peripheral blood can be below ten microorganisms per milliliter in adults and about 100 microorganisms per milliliter in children. In principle, blood cultures are sensitive enough to detect these low amounts of microorganisms; however, density varies during the course of disease, and therefore, blood culture diagnostics will not always yield positive results. Culturing sufficient quantities of blood is therefore recommended in patients with suspected sepsis [4].

Blood cultures have several advantages. First, they have been in use for more than 100 years and are well integrated in the clinical workflow and clinical guidelines. Second, semi-automated culture systems have greatly simplified handling in the microbiological laboratory which results in a short hands-on time. Third, a wide range of bacterial and fungal pathogens can be isolated and identified. Furthermore, isolation of the pathogen is a prerequisite to phenotypic susceptibility testing which enables clinicians to initiate targeted antimicrobial therapy.

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However, blood culture diagnostics has limitations: detection is limited to pathogens that are able to grow in blood cultures. Some microorganisms, such as *Legionella* spp., *Bartonella* spp., and *Aspergillus* spp., grow poorly in blood culture medium. Furthermore, antimicrobials may inhibit growth and relevant pathogens may thus go undetected, after the initiation of antimicrobial therapy. Most importantly, however, blood culture diagnostics requires some time until results are available and many clinicians feel that results are available too late to guide therapy.

Molecular techniques

Molecular techniques have been developed with the aim to improve sensitivity and to detect bloodstream infection earlier [5]. In pneumococcal pneumonia, blood cultures often remain negative and polymerase chain reaction (PCR)-based detection of *Streptococcus pneumoniae* in patients with a clinical suspicion for pneumonia has been shown to be more sensitive than blood cultures in clinical studies [6, 7]. However, in a recent meta-analysis of 29 studies, published between 1993 and 2009, the authors concluded that PCR from blood samples for the diagnosis of invasive *S. pneumoniae* infection lacks the sensitivity and specificity necessary for clinical practice [8]. This meta-analysis highlights the problems associated with determining accuracy of a diagnostic test. Most importantly, the lack of a good reference standard to diagnose invasive pneumococcal disease may have led to underestimation of test performance for PCR. All but three studies were performed retrospectively on stored frozen samples which may have impacted sensitivity. Furthermore, since all methodology was developed in-house, results may not be comparable between studies.

Detection based on broad-range PCR

In a review article from 2004 [9], Peters et al. looked optimistic upon the future of broad-range PCR assays which had been developed for the universal detection of bacteria or fungi deoxyribonucleic acid (DNA) in whole blood. They appreciated the enormous potential of PCR based amplification of 16S or 18S ribosomal ribonucleic acid (rRNA) genes that in principle allows detecting any bacterial or fungal DNA present in blood. Furthermore, different methods such as capillary sequencing analysis, pyrosequencing, or hybridization with specific probes can be used for identification. Although interpretation of re-

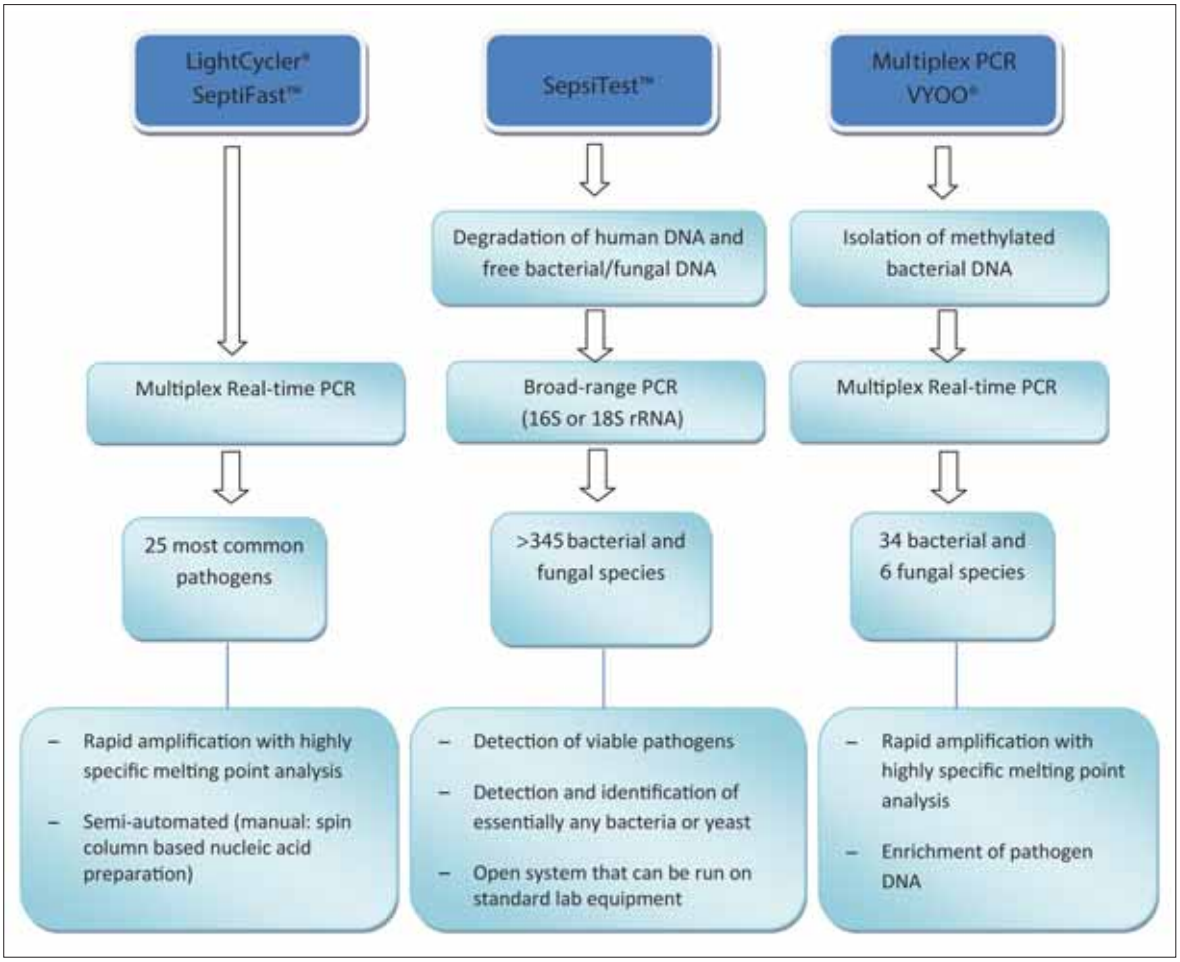

Fig. 1. Work flow for molecular techniques from the whole blood

Table 1. Studies with more than 80 adult patients enrolled that compare LightCycler Septifast™ (LCSF) with conventional blood culture (BC). † denotes studies where the biomarker procalcitonin (PCT) was used in patient allocation. ICU intensive care unit, PPV positive predictive value, NPV negative predictive value

Study	Sample size	Test performance	Conclusion
Paolucci et al. 2013 [41]	201 Neutropenic patients	71 Patients with positive LCSF or BC (concordance 79%). LCSF sensitivity 52%, specificity 89%, PPV 59%, NPV 86%	Results for LCSF were available earlier (median 3 days) than for BC, but BC is still indispensable due to false negative results
Mencacci et al. 2012† [42]	1009 Patients	In 136 patients 63 pathogens were detected by both methods, 48 only by LCSF, and 29 only by BC	PCT cutoff of ≥0.37 ng/ml is useful in febrile patients to predict LCSF positivity
Guido M et al. 2012 [43]	166 Neutropenic patients	In 40 patients, 23 positive BC, 38 positive PCRs. Concordance 53%. LCSF sensitivity 91%, specificity 88%, PPV 55%, NPV 98%	LCSF showed improved detection of slow growing organisms and in patients under antimicrobial therapy
Pasquallini et al. 2012 [44]	391 Patients	In 85 patients 60 pathogens were detected by LCSF and 57 by BC. LCSF vs. BC sensitivity 71% vs. 67%, specificity 100% vs. 94%, PPV 100% vs. 75%, NPV 92% vs. 91%	Combination of LCSF and BC significantly improved the diagnostic yield, esp. in patients under antimicrobial treatment
Rath et al. 2012 [45]	170 Patients	80 Samples positive BC, 92 samples positive LCSF. Sensitivity of LCSF 71%, specificity 76%, PPV 62%, NPV 83%	LCSF showed higher detection rates in liver transplant patients than in patients with major abdominal surgery (52% vs. 31%)
Lodes U et al. 2012† [46]	148 Samples in 104 patients	LCSF detected 77 pathogens in 59 samples. 30 of 77 pathogens detected by LCSF were not matched by conventional microbiology, however, PCT was high	In 25 episodes LCSF results changed antimicrobial therapy
Josefson P et al. 2011 [12]	1093 Patients on antibiotics with community-onset bloodstream infections	BC or LCSF were positive in 197 patients (18%). 50 concordant results. 64 samples positive only by LCSF, 86 only by BC. NPV for LCSF 97% for detectable pathogens	Low sensitivity (esp. for detecting *S. pneumonia*) suggests little advantage in diagnosing community-onset bloodstream infection
Bravo et al. 2011 [47]	86 Patients, 33 neutropenic, 53 ICU patients	LCSF sensitivity 62% in neutropenic patients and 70% in ICU patient. Concordant results in 69% neutropenic and 75% ICU patients. LCSF failed to detect 6 significant pathogens	LCSF results available after 7h; antimicrobial therapy could have been adjusted in 10 of 13 evaluated cases (retrospective analysis)
Maubon et al. 2010 [48]	110 Patients with cancer and sepsis	In 50 patients 55 pathogens were detected. LCSF vs. BC sensitivity 51% vs. 47%, specificity 83% vs. 95%, PPV 74% vs. 90%, NPV 69% vs. 66%	LCSF could have impacted antimicrobial treatment in 13% of patients (retrospective analysis)
Yanagihara et al. 2010 [49]	407 Samples in 212 patients	LCSF detected pathogens in 11.3% of samples (8.0% by BC). 23 pathogens were detected by LCSF only; LCSF missed five episodes of clinically significant bacteremia	LCSF offers added diagnostic value particularly in antibiotic pretreated patients
Avolio et al. 2010 [50]	144 Patients	In 53 patients with a pathogen detect by LCSF or BC, sensitivity of LCSF (assuming BC as standard) 91%, specificity 90%, PPV 75%, NPV 97%	LCSF can support BC in the early diagnosis of severe bloodstream infections
Varani et al. 2009 [51]	100 Immuno-compromised patients	Concordant samples in 79.2% of cases, LCSF failed to detect 5 cases of positive by BC	LCSF can be used in conjunction with BC but not replace it
Westh et al. 2009 [11]	558 Samples from 359 patients	231 Pathogens by LCSF or BC. 96 pathogens detected by BC (22 considered contaminants), 186 by LCSF (12 contaminants), 18 detected by BC only	LCSF detected more infections than blood culture (26% vs. 17%)

sults can be complex, due to detection of DNA rather than living pathogens, the risk of interfering contamination, the presence of human DNA in blood, and the lack of a gold standard, the authors expected that molecular assays will eventually replace the current conventional microbiological techniques for detection of bloodstream infections.

Almost a decade later, molecular techniques have not replaced blood cultures and it is still not clear how to best integrate them in diagnostic pathways. The reasons are manifold, e.g., only three tests are currently commercially available and clinical studies that show a clear advantage of molecular techniques in the clinical setting are lacking for all of them *(Fig. 1)*. In fact, in all studies, the lack of a good reference standard hampers interpretation of results. Further reasons are the costs for the test kits, the necessary equipment, and the availability of highly trained technical personnel. Convincing analyses of the costs and cost savings due to more rapidly available test results have not been performed. When these obstacles are overcome, physicians need to be convinced of the added value of molecular diagnosis, which can only be achieved when clinical studies show a clear benefit.

Lightcycler® Septifast™

One of the commercial tests available for the detection of microbial DNA in whole blood samples is the Light-Cycler® Septifast™ (LCSF) test (Roche Molecular Systems, Switzerland). It was designed to detect the 25 microorganisms that cause approximately 90% of all bloodstream infections [10].

There are quite a few studies that compare LCSF to conventional blood culture. Characteristics and results of studies that enrolled more than 80 adult patients are listed in *Table 1*. In the first study published in 2009 [11], 558 paired samples from 359 patients were evaluated. Of the 174 clinically relevant microorganisms identified with LCSF, 50 (29%) were also detected by blood cultures. More than half of the remaining microorganisms identified with LCSF (but not isolated after blood cultures) were also found in routine cultures of other relevant samples taken from the patients. There were 24 cases where LCSF did not detect clinically relevant pathogens; six of these were not included in the LCSF microorganism panel. The time to result was less than 6 h.

All subsequent studies *(Table 1)* showed similar characteristics as the first study: LCSF had a higher sensitivity than blood culture, especially in patients that had received antimicrobial therapy. Furthermore, the time to result was considerably shorter than in conventional diagnostic pathways. However, in every study, a significant proportion of clinically relevant pathogens were not detected by LCSF (false negatives) although they were included in the panel. The rate of false negative results ranges from 8% to 24%. In the study of Josefson et al. [12], the rate of false negative results for LCSF approached 50%. A possible explanation is that blood was kept until LCSF analysis at 4 °C for up to 3 days.

Studies that enrolled children *(Table 2)* did show a greater sensitivity and specificity of LCSF than studies in the adult population, presumably due to higher amounts of circulating pathogens in the bloodstream. However, false negative results still occur, and blood culture remains indispensable also in this population.

SepsiTest™

A commercially available test that uses broad range 16S and 18S rRNA gene analysis is SepsiTest™ (ST) (Molzym, Germany). Sample preparation from whole blood differs from other tests: human cells are lysed, and released human DNA as well as free bacterial or fungal DNA is degraded by DNAse before pathogens are subjected to lysis [13]. Pathogen DNA is then concentrated on a column, and three primer pairs are used to amplify Gram-positive, Gram-negative, and fungal rRNA. After DNA sequenc-

Table 2. Studies that have enrolled infants or children comparing LightCycler® Septifast™ (LCSF) with conventional blood culture (BC)

Study	Sample size	Test performance	Conclusion
Kasper et al. 2013 [52]	46 Preterm infants	In 15 positive cases sensitivity of LCSF vs. BC was 91% vs. 71%, specificity 80% vs. 100%, PPV 79% vs. 100%, NPV 90% vs. 81%	LCSF can rapidly detect nosocomial sepsis in preterm infants using a modified DNA extraction protocol
Tschiedel et al. 2012 [53]	110 Samples of 75 children	26 Pathogens detected by LCSF, 19 by BC. 14 samples positive by LCSF but negative by BC. Time to result for LCSF 6–17 h, 48 h for BC	LCSF detected pathogens earlier and more frequently in 97 patients that had received antimicrobial treatment (25%. Vs. 11%) than BC
Torres-Martos et al. 2012 [54]	42 Samples from 35 infants	LCSF sensitivity 79%, specificity 87%. Contaminants more frequent in BC vs. LCSF (16% vs. 2.4%)	LCSF is rapid (7h) and useful tool in combination with BC.
Lucignano et al. 2011 [55]	1673 Samples in 803 children	226 Pathogens detected by LCSF, 160 by BC. LCSF detected 97 additional pathogens, BC 27 additional pathogens	Higher specificity and sensitivity of LCSF than BC (LCSF 14.1% vs. BCs 6.5%)

ing, microorganisms are identified by an online BLAST search. The manufacturer recommends strict genus and species identification rules, and more than 97% identity in a read of at least 250 base pairs is required for reliable identification.

In the first clinical study using ST on 187 patients, 25 patients were identified where ST provided data on pathogens that were regarded as possible or true bloodstream infections [14]. In seven samples, ST did not detect relevant pathogens that were grown from blood culture (false negatives). In another study on 30 patients that underwent surgical valve replacement due to presumed infective endocarditis [15], microorganisms were detected by ST from heart valves and whole blood. ST did identify more patients than conventional culture (sensitivity 85% vs. 45%), but the study did not report the individual performance of analyzing whole blood. The lower sensitivity of culture was probably due to growth inhibition by antimicrobials. A further study evaluated 84 patients, 83 orthopedic samples, and 21 specimens from other normally sterile body sites, by ST and conventional culture [16]. ST detected more pathogens than conventional culture (34.6% vs. 25.0%). This was mainly due to the presence of fastidious or non-culturable species as well as previous antimicrobial treatment. Furthermore, ST was able to detect all culture-proven polymicrobial infections. The two studies suggest a role of ST in detecting infections from samples other than whole blood.

In our own experience, ST from whole blood detects more clinical relevant pathogens than blood cultures [17]. However, the reported times to result of 8 h [15] seem to be overly optimistic. In our hands, at least 10 h are required from drawing blood to the final report with a hands-on time of around 4 h. When expert personnel is not available, e.g., during weekends and holidays, the time to result can be much longer. A key issue in using ST is that an experienced microbiologist is needed who can minimize potential sources of contamination. Also, a high degree of suspicion is required when uncommon or unexpected pathogens are diagnosed by molecular methods, since clinical consequences can be serious. Furthermore, interpretation of sequencing results can be challenging when several sequences are overlapping due to polymicrobial samples. A commercially available software RipSeq (iSentio AS, Paradis, Norway) aids in interpretation of chromatograms containing up to three different bacterial species [18, 19].

VYOO® assay

The VYOO test system (SIRS-Lab, Jena, Germany) exploits differences in methylation between pathogen DNA and human DNA to enrich pathogen DNA from whole blood by affinity chromatography [20]. Pathogens are detected by a 16S rRNA gene multiplex PCR. The overall turnaround time is approximately 8 h. In a study on 72 patients, concordance of a microbiological workup (mainly blood culture) and VYOO® test (VO) from whole blood was 46.2% [21]. VO was not able to detect clinical relevant pathogens in eight cases. In a second study, concordantly positive results were achieved in 27 (8.7%) of samples, whereas 199 (64.0%) samples were negative in both tests [22].

Comparison of different molecular methods

In a small prospective observational study on 50 critically ill patients, ST, VO, and LCSF were compared to a constructed gold standard that included all clinical and microbiological information [23]. A positive predictive value (PPV) of 61.5% and a negative predictive value (NPV) of 83.8% was measured for blood cultures; for ST, a PPV of 100% and a NPV of 84.1%; for VO, a PPV of 100% and a NPV of 82%; and for LCSF, a PPV of 71.4% and a NPV of 81.4%. In this study, all three molecular assays outperformed conventional blood culture in respect to NPV and PPV. However, sensitivity was highest in blood cultures with 62% (ST 46%, VO 38.5%, and LCSF 38.5%).

In another small study on 57 critically ill patients [24], sensitivity and specificity as compared to conventional blood cultures taken from patients on two different occasions with at least 5 days apart were higher in LCSF (42.9% and 88.2%, respectively) than in ST (28.6% and 85.3%, respectively). In this study, specificity of LCSF was the highest, but this may have occurred due to the fact that 16 of 25 patients with a positive sample in any method were on antimicrobial therapy. Furthermore, 10 false positive results by ST due to contaminants highlight the importance of good laboratory practice for this method.

Biomarkers

Biomarkers, such as C-reactive protein or procalcitonin, aid in the diagnosis of sepsis and they are usually available before microbiological test results. Unfortunately, currently available biomarkers have a low sensitivity and specificity. As a consequence, in the early phase of bloodstream infection, diagnostic uncertainty is common and results in delayed treatment or overuse of antimicrobials [25, 26]. An ideal biomarker for bloodstream infection would have a high diagnostic accuracy especially early in the course of disease [27] and help in therapeutic decision making, screening, and diagnosis [28]. Furthermore, biomarkers should allow monitoring the patient's response to therapy at low cost [29].

C-reactive protein, an acute phase protein, and the white blood cell count are the most commonly used biomarkers for infection. However, both of them may be elevated in patients with autoimmune disease, cancer, tissue necrosis after ischemic heart attack, and severe virus infection [30]. Therefore, procalcitonin (PCT) is now widely used to presumptively diagnose severe bacterial infection

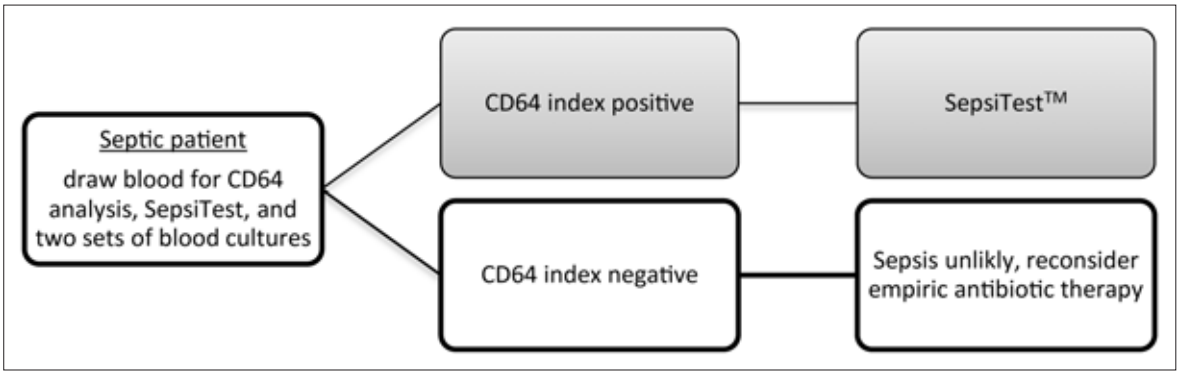

Fig. 2. Proposed clinical pathway for diagnosing bloodstream infection integrating molecular techniques and biomarkers

and sepsis. It is a ubiquitously produced precursor of the hormone calcitonin that is released upon endotoxin exposure and has demonstrated superior diagnostic accuracy for a variety of infections, including sepsis [31]. Furthermore, it allows monitoring the success or failure of antimicrobial therapy [32]. A recent review showed a remarkable influence on the initiation and duration of antimicrobial therapy when PCT was added to an antimicrobial stewardship program [33]. However, PCT may be elevated in patients with non-infectious disease conditions like severe congestive heart failure, acute pancreatitis, viral and parasitic infection [34–36].

A recent addition to the panel of biomarkers in sepsis is the expression of CD64 on polymorphonuclear leukocytes (neutrophils), which can be measured by flow cytometry. CD64 is a receptor molecule that recognizes the Fc portion of immunoglobuline G (IgG) and is constitutively found on macrophages and monocytes. Treatment of polymorphonuclear leukocytes with mediators of inflammation such as interferon-gamma and granulocyte-colony stimulating factor (G-CSF) induces CD64 expression within 2 to 4 h. This change is also observed in patients in response to bacterial infection [37, 38]. A recent meta-analysis concluded that the expression of CD64 has the highest diagnostic value of all bacterial infection biomarkers currently available [39].

Clinical pathways

To the best of our knowledge, there have been no studies that prospectively evaluated the impact of molecular techniques on clinical decision making and outcome. Furthermore, the benefit of rapidly initiating antimicrobial therapy may not be measurable in certain infections [40]. Therefore, it is unclear how to best integrate molecular techniques into clinical pathways. As a possible clinical algorithm, we propose using molecular techniques on whole blood of patients with elevated expression of CD64 on neutrophils, since these patients have a higher pre-test probability of a bloodstream infection *(Fig. 2)*. Additionally, conventional microbiological diagnostics needs to be performed to achieve a higher sensitivity and to isolate strains for susceptibility testing.

Conclusion

Molecular diagnostics have slowly started to change the approach of diagnosing bloodstream infection. Previous work has explored strengths and limitations of molecular methods and has strengthened the view that conventional microbiology is currently indispensable. It is now time to evaluate integration of molecular methods as add-ons to conventional microbiological techniques in clinical pathways.

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