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REVIEW

The challenge of molecular diagnosis of bloodstream infections

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Abstract
Early detection and identification of pathogens in bloodstream infections (BSI) is important to initiate or adjust antibiotic therapy as soon as possible. The current gold standard for diagnostic of BSI infection is the blood culture, that has a turnaround time of one to few days. Molecular tests performed directly in blood samples have promised faster diagnostics, with response times of a few hours, but their implementation into the clinical routine has been hampered by critical technical and procedural problems. Assay integration into laboratory workflows with random-access loading mode and minimal hands-on time is essential to meet rapid response times. Decreasing assay costs will favor fair clinical evaluations and might increase the applicability of the assays. Control of background contamination with bacterial DNA is one of the most difficult problems and might be avoided with pathogen-specific real-time PCR designs oriented to particular patient groups, or perhaps by quantitative, next-generation sequencing approaches.

Keywords Bloodstream infection · Sepsis · Molecular assays

The concept of bloodstream infection (BSI) is defined by the presence of bacteria or fungi in blood defined by positive blood cultures. Sepsis is a clinical syndrome elicited by a BSI and septic shock is a condition in which the patient shows refractory condition to specific therapeutic measures (Martinez and Wolk 2016; Viscoli 2016). These definitions are at the core of a complex diagnostic challenge because being a severe, potentially fatal, condition, the clinical suspicion of sepsis and septic shock demands prompt action (minutes to hours) (Gauer 2013), while the microbiological confirmation usually takes 1–5 days (Fernández-Romero et al. 2014; Tabak et al. 2018). Diagnosis is further complicated because a broad range of bacterial and fungal species may be responsible for BSI. In addition, the clinical definition is poorly specific, and the septic response may be due to viral infections or other non-infectious factors. Sepsis and septic shock are severe conditions, associated to high morbidity and mortality, increased hospitalization time and associated costs, and an early diagnosis would have a positive clinical impact on all these aspects (Buehler et al. 2016; Timbrook et al. 2017; Candel et al. 2018).

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The problem

The immediate response to the clinical suspicion of sepsis or septic shock involves support measures and empirical antimicrobial treatment. The etiology of BSI is broad, and the gold standard for microbiological diagnosis is the blood culture: blood culture bottle sets are inoculated, ideally before starting antimicrobial treatment, and loaded into automated monitoring systems (Lamy et al. 2016). In general, positive results (including identification and antimicrobial susceptibility testing) take 2 to 6 days for bacteria, and somewhat longer for yeasts, while negative results are reported after 5 days (unless there is a suspicion of fastidious zoonotic agents). During the last years, the response time, once a bottle becomes positive, has been reduced by the use of MALDI-TOF mass spectrometry for pathogen identification in samples taken directly from the positive bottles. If a single pathogen is identified, and taking into account the available data on the focus of infection, direct susceptibility testing can be done with the same samples (Romero-Gómez et al. 2012; Faron et al. 2017; Chandrasekaran et al. 2018; Périllaud et al. 2018). But in spite of these improvements, the time from inoculation to positivity still depends on the growth rate and the initial load of the pathogen.

Several host biomarkers have been developed in order to measure them directly in blood and give faster responses.

Some of them, like C reactive protein or procalcitonin (Prucha et al. 2015), have been incorporated into the standard clinical procedures, but most have shown limited sensitivity or specificity, and there is not a unique reliable marker of infection for all patient populations. Several sepsis scores have been developed combining different parameters and have been validated for specific patient populations (van der Geest et al. 2016; Kuzniewicz et al. 2016; Shane et al. 2017).

The limitations of molecular approaches

An obvious alternative to accelerate the diagnostic would be the development of fast molecular techniques to detect microbial DNA directly in blood samples. Several assays have been developed and some of them have been commercialized (Opota et al. 2015; Martinez and Wolk 2016; Ginn et al. 2017; Florio et al. 2018; Peker et al. 2018; Sinha et al. 2018), but so far none of them has succeeded in entering into the clinical routine for a variety of reasons, either technical and methodological.

A common shortcoming comes from difficulties in the adaptation of the assays into laboratory workflows (Buehler et al. 2016). For a laboratory working on a single shift, an assay lasting 4 to 6 h may not be considered a rapid technique because many samples (i.e. those that arrive towards mid-shift or later) will be left for the next day extending the average response time to 1 day (Fernández-Romero et al. 2014). This would not be the case for simple assays, i.e. those called point-of-care assays, or for fully automated methods that might be set up at any time, even by non-specialized staff during the evening and night shifts. So, while the reporting times of point-of-care or fully automated assays might be shorter than those of blood cultures, those of manual or partially automated assays might have little advantage, particularly if they have to be batched, or fitted into the workflow of single shift laboratories. Successful assay designs must take into account the assay response time, including the hands-on time and the running modes (i.e. on-demand versus batch), and their integration into different laboratory workflows and working times.

A common handicap found by several rapid assays has been the unfavorable design of clinical evaluations, mostly due to the high cost per test. So, commercial molecular assays have been usually assessed using a single, small volume, blood sample and comparing the results with those of a whole set of blood culture bottles (2–3 bottles, inoculated with up to 20–30 ml of blood) (Dark et al. 2012; Herne et al. 2013; Fernández-Romero et al. 2014). Even under these disadvantageous conditions, the sensitivity and specificity of the molecular assays have been shown to be good and have compared favorably with blood cultures. Performing the molecular assays on two or more samples taken from different sampling sites or at different time points would further increase their sensitivity and specificity. Along this line, follow-up studies have been done assaying serial samples to monitor response to the therapy in patients that have been already diagnosed (Fernández-Cruz et al. 2013; Falces-Romero et al. 2018a). These strategies expand the role of molecular assays beyond diagnostics but their implementation into routine practice is limited by costs.

A third important limitation results from the nature of BSI and the design of the assays. The etiology of BSI is broad (Akova 2016; Bassetti et al. 2016; Del Bono and Giacobbe 2016; Martinez and Wolk 2016; Viscoli 2016; Yahav et al. 2016), about 90% of the cases are produced by 10–20 different pathogens: Gram-positive cocci, *Enterobacteriaceae*, *Pseudomonas* spp., *Acinetobacter* spp. and *Candida* spp. Yet there is a significant percentage that is produced by a large variety of infrequent (Reigadas et al. 2015) or sporadic microorganisms (Sharara et al. 2016; Falces-Romero et al. 2018b; Gross et al. 2018; Alguacil-Guillen et al. 2019). One approach to detect any potential pathogen is to target assays to essential, broadly conserved sequences using so-called "universal" primers or probes (mostly directed to the 16S rRNA genes for bacteria and the 18S rRNA genes or ribosomal operon ITS sequences for fungi). The weak point is that reagents often contain minute amounts of bacterial DNA that will also be detected by the assays (Corless et al. 2000; Czurda et al. 2016). Furthermore, blood pathogen loads are frequently low (Yagupsky and Nolte 1990) and the assays require extremely clean, i.e. DNA-free, reagents and working environment and procedures to avoid high rates of false positives. This problem has proven quite difficult to solve in practice, leading some companies to target assays to positive blood culture bottles (Peker et al. 2018), to develop pathogen-enrichment methods (Pilecky et al. 2018), or to design pathogen-targeted assays that use specific probes and primers instead of "universal" ones. The later approach has been further developed into "syndromic panels" that are being successfully used for some types of infections (i.e. respiratory or gastrointestinal panels) (Ramanan et al. 2017). For BSIs the limited number of pathogens tested has been critical because the risk of false negative results severely compromises the reliability of the assays and has limited their commercialization potential.

The challenge

The obvious challenge is to design rapid and cost-effective molecular assays with high sensitivity and specificity. A favored strategy is based in closed-tube, non-contaminating, real time PCR approaches, targeting the most common pathogens for selected groups of patients with well-defined sepsis etiologies (Fernández-Romero et al.

2014; Zacharioudakis et al. 2018). Additionally, panels for specific patient groups might be custom-designed based on the local epidemiology. These might include also the most relevant resistance determinants (for example *mecA* gene or carbapenemase genes), and ideally should include "universal" markers to ensure detection of infrequent, non-targeted pathogens, even if these have to be identified later with other techniques (sequencing, blood culture). New developments might take advantage of the nature of real-time PCR to go beyond diagnostics, and make use of the small sample volumes required and the quantitative potential of the assays (Ct values) to monitor patient response to treatment (Liu et al. 2012; Ziegler et al. 2014).

Sequence-based approaches are promising hypothesis-free alternatives. PCR amplification and sequencing of bacterial or fungal rRNA genes with "universal" primers may identify any pathogen, even new ones. Classical Sanger sequencing is not a rapid technique, but pyrosequencing is fast enough for rapid applications (Morinaga and Yanagihara 2015), nevertheless, development of commercial systems based on sequencing has been hampered by the problems associated to open-tube PCR methods and DNA-contaminated reagents in samples with low or very low pathogen loads, in which the amounts of target sequences may be similar to those of microbial DNA found on the negative controls (Fenollar and Raoult 2007; Philipp et al. 2010; Eisenhofer et al. 2019). Next-generation sequencing approaches might be used to sequence pathogen DNA from blood without previous amplification, either directly (Grumaz et al. 2016) or after enrichment of non-human DNA (Hewitt et al. 2018), though these approaches are still far from practical routine application (McAdam 2018) and are not free of the problems of low pathogen load samples (Eisenhofer et al. 2019). A major advantage of next-generation sequencing is that it is not restricted to bacterial pathogens but will also detect and identify fungi (Hong et al. 2018) or viruses (Suzuki et al. 2017), and might give quantitative results by measuring depth and coverage.

In summary, there is an unmet need for faster and better diagnostic methods for BSI/sepsis directly from blood samples. Some of the candidate technologies are still in development, some others are already mature, though there is still a need for DNA-free reagents and contamination-proof assays designed to fit into the clinical microbiology laboratory workflows.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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