**Abstract**

Blood-stream infection (BSI) by microorganisms can lead to sepsis. This condition has a high mortality rate, which rises significantly with delays in initiation of appropriate antimicrobial treatment. Current culture methods for diagnosing BSI have long turnaround times and poor clinical sensitivity. While clinicians wait for culture diagnosis patients are treated empirically, which can result in inappropriate treatment, undesirable side-effects and contribute to drug resistance development. Molecular diagnostics assays that target pathogen DNA can identify pathogens and resistance markers within hours. Early diagnosis improves antibiotic stewardship and is associated with favourable clinical outcomes. Nonetheless, limitations of current molecular diagnostic methods are substantial. This article reviews recent commercially available molecular methods that use pathogen DNA to diagnose BSI either by testing positive blood cultures or directly testing patient blood. We critically assess these tests and their application in clinical microbiology. A view of future directions in BSI diagnosis is also provided.

**Introduction**

The presence of infectious organism in the blood, including bacteria (bacteraemia), viruses (viraemia) or fungi (fungaemia), is generalised as blood stream infection (BSI). The prevalence of BSI is on the increase owing to factors such as immunosuppression treatment, increased frequency of invasive procedures, aging population and rise of multidrug resistant organisms in hospitals and care homes [1]. BSIs may be associated with infections such as endocarditis and procedures such as central venous catheter, colonoscopy or dental extraction. BSIs may also be caused by secondary focal infections such as pneumonia, urinary tract infections or abscesses [2] . BSI often develops to sepsis (or severe sepsis), which can be defined as a systemic response to infection with the presence of some degree of organ dysfunction. It can be categorised as septic shock when complicated by either hypotension that is refractory to fluid resuscitation or by hyperlactatemia [3-6]. In Europe and US combined sepsis causes more than 400,000 deaths every year, costing an estimated $17 billion in the US [7-10].

The high mortality rate associated with sepsis is influenced by a combination of factors including the characteristics of the infecting pathogen and the host response to infection. Recent studies suggest that every hour delay in initiation of effective antimicrobial therapy in patients with septic shock is associated a decrease in survival rate [11,12]. The Surviving Sepsis Campaign recommends confirming sepsis by objective evidence of infection, which is achieved by identifying live pathogens by blood culture methods. Culture analysis, including drug susceptibility testing, takes at least 48 hours for positive samples and 5 days to identify specimens as negative [3,11].

Because of the temporal separation between initial clinical suspicion of sepsis and laboratory confirmation of BSI, a ‘safety first’ strategy of using potent broad-spectrum antibiotics within 1h of onset of sepsis is employed, aimed at covering the most probable pathogens [3,13]. Although effective [13], rapid empirical treatment of septic patients has several limitations: (i) under-treatment of a few patients with resistant pathogens, with contingent mortality (ii) over-treatment of considerably more patients with susceptible pathogens, with antibiotic over-use and increased cost and occurrence of adverse drug effects [13,14] and; (iii) the clinical utility of blood samples taken for blood culture after antibiotic therapy is administered can be diminished due to sterilisation of the sample [3]. Guidelines also state that antibiotic treatment should be tailored following microbiological culture results [3]. Benefits of tailored treatment include: use of less toxic agents; use of antibiotics that achieve higher concentrations; the option to select more potent agents if necessary; and it is associated with favourable clinical outcome [15,16]. However a recent study by Garnacho-Montero showed that 12% of patients die before microbiology results are available to clinicians [15]. Additionally, culture is, at best, only 60% sensitive [10,17-19] and, if no pathogens are grown, there is no scope to guide the refinement of treatment. The slow turnaround time of culture methods means prolonged use of broad-spectrum antibiotics even if a pathogen can be grown and identified. Extended use of broad-spectrum antibiotics is associated with increased antibiotic resistance and collateral damage to the normal gut flora resulting in colonisation by opportunistic pathogens such as *C. difficile* and/or the selection of Enterobacteriaceae with transferrable drug resistance [20,21].

A number of new technologies have been introduced that speed up phenotypic diagnosis, such as automated continuous culture systems, rapid pathogen identification using MALDI-TOF and automated systems for antimicrobial susceptibility testing. While progress has been made, none of the methods have succeeded in changing clinical practice [22,23]. The speed and accuracy of new molecular diagnostics assays have potential to improve the current situation. Rapid results available to clinicians before a second dose of broad-spectrum treatment is administered (6-8 hours) would result in improved antibiotic stewardship and reduced patient morbidity and mortality.

Currently, detection of pathogen DNA in blood by molecular methods relies on nucleic acid amplification by real-time PCR with or without a period of incubation in blood culture. Because of the large and diverse range of pathogens that cause sepsis, most PCR assays have been multiplexed. Multiplexed and broad range assays have been designed to cover most likely pathogens and, in some cases, important antibiotic resistance markers. In this article we review the commercially available molecular assays for the detection of pathogen DNA either from positive blood culture or directly from whole blood.

**Commercially available methods for pathogen detection in positive blood cultures**

***Prove-it™ Sepsis***

Mobidiag® (Finland) have developed the Prove-it™ Sepsis assay designed to shorten the time to identification of pathogens and their resistance genes in positive blood culture bottles. The technology is based on broad range PCR targeting conserved regions of topoisomerase genes, gyrB and parE [24,25], followed by microarray based detection using genus and species specific probes. Biotin-labelled PCR amplicons bind to the DNA probes on the microarray and a colorimetric output is derived by horseradish peroxidase(HRP) induced tetramethylbenzidine (TMB) substrate based precipitation staining of the biotin-labelled PCR product. The pathogen/resistance marker present in the sample is identified using purpose built software. The Prove-it™ platform can process between one and 96 samples at a time, and identify pathogens from positive blood culture in 3 hours [25].

The first version of Prove it™ Sepsis, could identify more than 50 species of Gram-positive, Gram-negative bacteria and the methicillin resistance marker *mecA*, however the panel did not include fungal targets. The performance of this version of the Prove-it™ assay was validated and evaluated by Tissari *et al* [25]. The study included 3318 blood samples from patients with clinically suspected sepsis of which 2107 yielded positive blood-culture [25]. The bacterial species in culture positive samples were identified by conventional culture and the Prove-it™ sepsis assay. 1807 of 2017 (86%) positive blood-culture pathogens were covered by the Prove-it™ assay and in this subset of samples the test was 94.7% sensitivity and 98.8% specificity for pathogen identification and 100% accurate in identifying MRSA. A limitation of the assay is that it is capable of detecting a limited range of pathogens, hence could not identify the organism present in 14% of positive blood cultures [25]. *Streptococcus viridans* and *Candida* species were the most frequent of the missed organisms. The main reported advantage of the test is that it provided results 18 hours faster, on average, than conventional culture. The recent introduction of MALDI-TOF, however, has reduced the turnaround time for conventional culture, hence the time advantage of the Prove it test may have been lost.

The current version of Prove-it™ Sepsis (StripArray v2.0) can identify 60 bacteria, 13 fungi and the *mec*A gene. This version has been extended to cover most clinically relevant *Candida* species, including *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. lusitaniae*, and *C. krusei*. Others are *C. dubliniensis*, *C. pelliculosa*, *C. kefyr*, *C. norvegensis*, *C. haemulonii*,and *Saccharomyces cerevisiae*.The use of the Prove-it™ V2 assay for identifying clinically relevant fungi in blood culture was validated in a study by Aittakorp *et al* [26]. A total of 388 specimens were analysed, including 160 blinded fungal clinical isolates, 69 blood cultures spiked with different candida species, 19 blood samples from patients with suspected fungal sepsis and DNA extracts from blood cultures positive for yeasts (44) and bacteria (16) and from negative blood cultures (82). The Prove-it™ Sepsis platform demonstrated sensitivity of 99% and specificity of 98% for fungal targets. Rapid and accurate diagnosis of fungemia is important so that treatment can be tailored for favourable patient outcomes [26]. A limitation of the fungal portion of the Prove-it™ test is that opportunistic fungal pathogens, such as Cryptococcus species, found in immunocompromised patients are not detected by the assay [26].

***FilmArray® blood culture identification panel***

The FilmArray (BioFire Diagnostics, USA) blood culture identification (BCID) panel consists of 24 target pathogens (8 genus/species of gram positive, 11 genus/species of gram negative, 5 species of candida) and 3 antibiotic resistance genes (*mecA, vanA/B* and KPC) associated with bloodstream infections. The FilmArray pouch contains all reagents necessary for sample preparation, amplification and detection; the user injects hydration solution and positive blood culture sample combined with sample buffer into the pouch. The FilmArray instrument first extracts and purifies nucleic acids from positive blood culture sample and then amplifies the target genes in a large volume, multiplex, reverse-transcriptase first stage PCR reaction. To detect products from first stage PCR, individual single-plex second-stage nested PCRs for all target genes are performed in individual wells. Amplification of the second stage PCR is monitored in real-time using a double stranded DNA intercalating fluorescent dye. Post PCR melt curve analysis is then performed to identify the specific pathogens and resistances. The FilmArray platform is a closed diagnostic system which requires 2 minutes hands-on time and about 1 hour turnaround time, however, only one sample can be analysed at a time [27].

In a study reported by Blaschke *et al* [28], the FilmArray BCID panel identified 95% of 109 pathogens detected by culture. Three pathogens identified by FilmArray were not identified by culture (one *S. agalactiae* misidentified as *S. aureus*; one *S. aureus* misidentified as CoNS and one *S. epidermiditis* negative by culture) [28] . Overall in 111 prospectively collected blood cultures the assay detected 84 of 92 (91%) of pathogens covered in a panel and 100% detection of MRSA and vancomycin-resistant Enterococci. Sensitivity for each pathogen ranged from 83% to 100% and the assay was 99% specific. However the BCID panel failed to identify 8 pathogens in 92 blood cultures positive for BCID pathogens, 3 of those missed contained two organisms, 2 were as result of primer cross-reactivity and 2 were samples positive for coagulase negative Staphylococci but negative for BCID pathogens and finally *S. marcescens* was identified by culture but not detected by FilmArray. FilmArray also missed 7 samples because the organisms detected by blood culture were not on the BCID panel [28].

The performance of the FilmArray BCID assay was also evaluated in paediatric blood cultures in a recent study by Zheng et al. The BCID panel identified 168 (89.4%) of 188 organisms in culture bottles [29]. Results showed of the 116 blood culture isolates that the BCID assay could detect, 112 (91.8%) were correctly identified. Detection of the *mecA* gene in 86 *Staphylococcus* species and *vanA*/*B* in 12 Enterococci was 100% accurate. The assay, however, failed to identify 20 organisms of which 13 organisms were not on the assay panel and 3 organisms that were in mixed cultures [29].

***Verigene® Gram-positive and Gram-negative blood culture tests***

Verigene® (Nanosphere Inc) is a commercially available microarray based system with panels for the identification of blood cultures containing Gram-positive bacteria (BC-GP test) or Gram-negative bacteria (BC-GN test). The BC-GP detection panel includes 13 species/genus targets and 3 resistance markers, *mec*A, *van*A, and *van*B [30]. The BC-GN panel targets 9 species/genus targets and 6 resistance markers, one for CTX-M (ESBL) and 5 markers for carbapenem resistance (*bla*KPC, *bla*NDM, *bla*VIM, *bla*IMP, and *bla*OXA) [31].

The Verigene system consists of sample processor and microarray reader. Nucleic acids are isolated from positive blood culture in single use extraction tray by magnetic beads extraction. The extracted nucleic acid is denatured and applied to the Verigene solid-phase microarray. The microarray consists of single-stranded, target-specific capture probes immobilised onto the surface of a glass slide [32]. If target DNA is present in the extracted nucleic acids, it will hybridise to the complimentary capture probe on glass slide microarray. Gold microspheres coated with single stranded nucleic acid complementary to a different region of the target are added to form a sandwich nucleic acid structure with the probe-target sequence. Detection of target template depends on this second hybridisation of nanoparticle-conjugated detection probe. Capture probes for each target along with internal controls are present in triplicate. The turnaround time to pathogen identification is approximately 2.5 hours [32].

Recently the Verigene BC-GN test has been evaluated on paediatric samples in a multicentre study. The BC-GN test was performed on blood cultures positive for Gram-negative organisms. A total of 104 organisms were isolated from 97 positive clinical blood cultures [31]. BC-GN correctly detected 26/26 cultures with *Acinetobacter* species, *P. aeruginosa*, and *S. marcescens*, 5/6 with *Citrobacter* species, 13/14 with *Enterobacter* species, 23/24 with *E. coli*, 2/3 with *K. oxytoca*, 16/17 with *K. pneumoniae*, and 0/1 with *Proteus* species. Overall the BC-GN test correctly identified the organism/s present in 52/57 (91%) blood cultures from one centre and 41/47 (87%) from the other centre. BC-GN was appropriately negative in 8/13 blood cultures that were not represented on the BC-GN microarray detection panel. Of the five cultures that grew multiple Gram-negative organisms, BC-GN failed to detect targets in three. The assay was 100% accurate for clinical blood cultures with blaCTX-M (5/5) and blaVIM (1/1) [31].

In another multicentre paediatric study, the Verigene BC-GP test was evaluated in 249 gram-positive organisms from 242 blood cultures. 106 *S. aureus* isolates were recovered. The BC-GP test analytical sensitivity was 100% for *S. aureus,* 99% for MSSA and 100% for MRSA [33]. Overall *S. aureus* specificity was 100%; 100% for MSSA and 95% for MRSA. For other organisms BC-GP sensitivity varied between 33% and 100%. 7 results of the 249 tests were reported invalid because of reasons including internal control failure and variation in target-specific signals. 2% of results were incorrect in one centre and 5% in the other. The proportion of pathogens that were not detected as they were not represented on the microarray panel was 4% in the first centre and 5% in the second [33]. Limitations highlighted by the authors include: the inability of the test to ascertain the source of antibiotic resistance genes when there is a mixed blood culture e.g. in cultures positive for *S. epidermidis*, *S. aureus* and *mecA,* it is impossible to ascertain from which organism the *mecA* originated; and the limited nature of the panel for identifying certain species e.g. coagulase negative staphylococci[33].

The findings from two additional studies have shown Verigene BC-GP can provide organism identification and susceptibility results approximately 42h earlier than conventional methods (using traditional pathogen identification) [30,34]. Beal et al suggest that the test could be used to guide appropriate antibiotic selection earlier and that contact isolation could also be initiated significantly earlier for patients with MRSA or VRE[34].

***Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI-TOF MS)***

The diagnostic advantage of molecular microarrays, hybridisation assays and PCR based methods for detection of pathogens in positive blood culture bottles (Table 1) will be challenged by the introduction of MALDI-TOF MS methods. MALDI-TOF methods have been widely accepted due to the low cost per sample analysed, simple protocol for sample preparation, rapid turn-around time and large database for species and genus identification [22].

The performance of the MALDI Sepsityper Kit (Bruker, Germany) on positive cultures was assessed in a number of studies. The correct species was identified in 80% (332 out of 358) of positive cultures in one study [35], and above 90% in another [36]. The VITEK Mass Spectrometry System (Vitek MS; bioMerieux, NC, USA) was evaluated against the FilmArray system using 151 mono-microbic isolates. The FilmArray correctly identified (98%) to the genus and (100%) to the species/complex level, while Vitek MS correctly identified (94%) mono-microbic cultures to the genus level, (91%) to species level [37]. FilmArray missed 18/151 (12%) blood cultures because they are not on identification panel but identified resistance genes with 100% sensitivity and specificity. Vitek MS missed no blood cultures but does not test for antibiotic resistance. Both platforms provided results within an hour [37]. MALDI-TOF is already integrated into the diagnostic pipeline in many clinical microbiology laboratories and, together with the simple, rapid, low-cost, comprehensive protocol, it is likely to be preferred over other platforms for identifying pathogens in positive blood cultures [36]. Detection of antimicrobial resistance using MALD-TOF is an active area of research including detection of antibiotic modifications by degrading enzymes, detection of resistance mechanism determinants through proteomic studies of multiresistant bacteria, and the analysis of modifications of target sites, such as ribosomal methylation [38-40].Studies in this area are comprehensively reviewed by Hrabak et al and they demonstrate that MALDI-TOF is a relevant tool for the detection of antibiotic resistance. MALDI-TOF can also be used together with automated antimicrobial susceptibility testing devices such as the BD Phoenix [41].

**Commercially available methods for pathogen detection directly in whole blood**

***T2Candida® test***

The T2Candida test (T2Biosystems, MA, USA) enables nanoparticle-mediated rapid detection of candidemia directly from whole blood. The T2Candida® assay based on T2MR technology can identify the 5 most clinically relevant species of *candida (C. albicans, C. tropicalis, C. parapsilosis, C. glabrata*, and *C. krusei)* directly from patient blood samples. T2Candinda® uses specific primers to PCR amplify candida DNA which then binds to paramagnetic nanoparticles coated with complementary probe. For each target, two nanoparticles are designed, each bearing a different complementary probe; one hybridizing to the 5′ end of the single stranded DNA target and one to the 3′ end. Upon hybridization, the nanoparticles cluster and the extent of clustering increases with target DNA concentration [42]. This clustering alters the magnetic resonance signal in the sample, indicating the presence of the target. The change in signal (T2 relaxation) is measured by the T2Dx® instrument - an automated sample-in, answer-out bench-top device. According to the manufacturer, the T2Candida test has a limit of detection of 1 colony-forming unit/ml and can be performed within 3 hours.

The performance of the T2 Candida test was evaluated on blood samples from three patients (21 blinded clinical specimens in total) collected serially. *C. albicans* infection was detected in all cases by both the T2 test and blood culture. One day after starting antifungal administration all blood cultures become negative while T2 test remained positive indicating the detection of dead cell DNA [42]. Healthy donor blood samples spiked with the 5 *Candida* on the panel showed 98% positive agreement and 100% negative agreement between the T2 test and blood culture. A limit of detection (LoD) of 3 CFU/ml was observed for *C. albicans* and *C. tropicalis*; an LoD of 2 CFU/ml was observed for *C. krusei* and *C. glabrata*; and an LoD of 1 CFU/ml was observed for *C. parapsilosis* [42].

The performance of the T2Candida® test was evaluated against blood culture in an independent study by Beyda et al. The five Candida species on the test panel were spiked in blood samples at concentrations ranged between 3.1 and 11 CFU/mL [43]. In a total of 90 samples tested, T2Candida test sensitivity was 100% and specificity was 98% (4 false positive results were reported). The molecular test results were reported with 3 to 5 hours compared to an average of 63 hours for BACTEC blood culture. The study did not report on the performance of the test on mixed samples [43].

Recently, the US Food and Drug Administration has approved the T2Candida® panel and T2DX® instrument for use directly in blood. T2 Biosystems is currently developing a T2Bacteria test, a multiplex panel for the detection of the major bacterial pathogens associated with sepsis. According to T2Biosystems, the T2Bacteria test will have very similar performance characteristics as the T2Candida test.

***LightCycler® SeptiFast assay***

The LightCycler Septi*Fast* assay (Roche Diagnostics, Germany) was the first commercial multiplex assay for the detection of sepsis pathogens directly from blood and hence is the most studied. The assay can detect and identify 25 of the most common bacteria and fungi directly from blood without prior incubation or culture. The panel includes Gram-negative organisms: *E.coli*, *Klebsiella (pneumoniae / oxytoca)*, *Serratia marcescens*, *Enterobacter (cloacae / aerogenes)*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*; andGram-positive organisms: *Staphylococcus aureus*, coagulase negative *Staphylococci* species*, Streptococcus pneumonia, Streptococcus species, Enterococcus faecium, E. faecalis*, five *Candida* species and *A. fumigatus* [44]*.*

The Septi*Fast* assay uses dual labelled fluorescent energy transfer probes (FRET) targeting species-specific regions of internal transcribed spacer (ITS). The ITS target sequences are located between the 16S and the 23S ribosomal DNA sequences of bacteria and between 18S and 5.8S ribosomal sequences of fungi [45]. DNA is extracted by mechanical lysis with ceramic beads using MagNa Lyser (Roche Molecular Systems). Extracted DNA is purified and three multiplex PCRs are set up (Gram-negative bacteria, Gram-positive bacteria and fungi). Rapid real-time PCR is run on the LightCycler® 2.0 instrument. Melt peak analysis of the PCR amplicons provides specific melting points that enable genus and species identification. The detection limit of the different targets in the assay range from 3 to 30 CFU/ml and the turnaround time is approximately 6 hours [45,46].

The LightCycler® SeptiFast assay has been evaluated in severe sepsis [47], paediatric patients [48,49], intensive care [20] and general medicine [50]. However different studies have shown different ranges of specificity and sensitivity. In a systematic review and meta-analysis Chang *el al* have extracted and pooled data from 34 primary studies (from 248 citations) that included (1) evaluation of Septi*Fast* diagnosis of sepsis (2) comparison of Septi*Fast* test results with reference standards, and 3) sufficient information to calculate sensitivity and specificity. A total of 8,438 suspected sepsis episodes (from 6,012 patients) were analysed of which 22.8% (1,920 episodes) were confirmed positive for bacteria or fungi. The overall sensitivity of Septi*Fast* to detect BSI was 75% and specificity was 92% [51]. For bacteremia sensitivity was 80% and specificity of 95% whereas fungemia had 61% sensitivity and 95% specificity [51]. Lower but comparable sensitivity and specificity results have been reported in a recent systematic review from 41 phase III diagnostic accuracy studies. Dark and colleagues have reported Septi*Fast* sensitivity of 68% and specificity of 86% compared to blood culture [52].

A study by Maubon et al evaluated the impact of Septi*Fast* in therapeutic decision making. The Septi*Fast* results would have significantly improved treatment in 11 (10%) patients and prompted immediate antimicrobial therapy not given initially in 3 patients. However, the test was reported to produce 10 false positive results, which would have resulted in unnecessary antimicrobial treatment in these patients.

***SepsiTest®***

SepsiTest (Molzym, Germany) is based on the universal or real-time PCR detection and sequence identification of organisms causing sepsis. The assay detects presence of organisms in the sample using PCR targeting the 16S rDNA gene of bacteria and the 18S rDNA gene of fungi. PCR results indicate possible presence or absence of bacteraemia and/or fungaemia. In positive cases, the amplicon is analysed by sequencing for definitive identification of the organism. The assay includes selective degradation of potentially PCR-interfering human DNA while leaving pathogen cells/DNA intact. This technology can detect more than 345 bacteria and fungi with an overall LoD of 20-460 CFU/ml [53]. First part of the assay can be completed within 4 hours however if the sample is positive sequencing is required and this extends turnaround time about by at least 8 to 12 hours [1].

In one multicentre study, SepsiTest® was evaluated in 342 blood samples from 187 patients with systemic inflammatory response syndrome, sepsis or neutropenic fever. Compared to blood culture the test had a sensitivity of 87.0% and specificity of 86% [54]. The assays detected polymicrobial infection in 12.5% patients compared to 7.4% by culture. Moreover the test detected obligate anaerobes and other organisms regarded as contaminants that failed to grow in blood culture. However, 7 of 54 samples of the study were blood culture positive but PCR negative. Of the 288 blood culture negative samples, 41 samples (from 31 patient) were positive by PCR and the PCR result was judged as possible or probable true bacteraemia in 25 of these patients. Accordingly, the PCR detection rate was 25.7% and the culture detection rate was 15.8%. Most of the PCR-positive, culture negative patients classified as probable or possible bacteraemia (17 of 25) received antimicrobial therapy before blood sampling. This suggests that the bacterial species detected by PCR may have been nonviable and thus not detectable by culture.[54]. The long turnaround time associated with positive results has led to limited use of the test. Nevertheless, diagnostic qualities such as high sensitivity and detection of non-viable cells can provide extra information, making this test useful as a supplementary diagnostic for sepsis.

***VYOO®***

VYOO® (Analytik Jena, Germany) is a multiplex PCR assay that specifically identifies 46 sepsis-related pathogens (99% of all sepsis-related pathogens) and resistance genes (34 bacteria, 7 fungi and 5 resistance markers). The assay involves mechanical lysis of whole blood, automated total DNA extraction and pathogen DNA enrichment (LOOXSTER® technology). The pathogen enriched DNA is amplified by PCR then run on agarose gel. Pathogen identification is possible through analysis of the pathogen-specific electrophoretic pattern (the most recent version of the test uses microarray based detection). Turn-around time is estimated to be 7 hours and the manufacturer claimed sensitivity is between 5 and 100 CFU/mL.

An observational study by Bloos *et al* compared the performance of the multiplex VYOO™ assay with blood culture on 311 blood samples from 245 patients with suspected sepsis. In total, 45 of 311 (14.5%) samples were blood culture positive and 94 of 311 (30.1%) were PCR positive [55]. 27 (8.7%) samples showed a concordantly positive in both tests while 199 (64.0%) were negative in both tests. 67 (21.5%) samples were PCR positive despite being culture negative; while 18 (5.8%) were PCR negative despite being culture positive. Overall sensitivity of VYOO to detect culture positive bacteria was 60% with 75% specificity [55]. When all the technical requirements were optimised, the VYOO assay turnaround time was 7.2 hours compared to 68.8 hours for positive blood culture and 191 hours for negative blood cultures [55].

Three commercially available PCR based platforms for the diagnosis of BSI (SepsiTest, VYOO and Septi*Fast*) were compared against blood culture in a prospective observational study. The study involved 50 clinically ill patients with sepsis, severe sepsis or septic shock [56]. Blood culture was positive for 13 samples (26%), but after considering all clinically relevant information only 8 were deemed relevant. No pathogen was identified by the three PCR methods in 5 of the 8 relevant positive blood samples. 32 (64%) samples were both blood culture and PCR negative. SeptiTest® identified 6 samples (12%) that were consistent with the findings of blood culture. The VYOO® assay identified pathogens in five samples (10%) of which four were considered relevant. However, of the eight relevant culture positive samples, the VYOO® was negative for all. Septi*Fast* identified 8 pathogens in seven samples; the test was negative in 5 of the 8 clinically relevant positive blood cultures. There were an unusually low number of PCR positive results observed compared to blood culture in this study despite 72% of patients receiving antibiotic treatment prior to sampling. A larger study is required to identify which test is superior [56]. The VYOO detection panel includes 5 resistance markers but the sensitivity and specificity of the test for these markers remains to be demonstrated.

***Magicplex™ Sepsis Real-time***

The Magicplex Sepsis Real-time test (Seegene, Korea)screens for more than 90 sepsis-causing pathogens from whole blood samples. The assay detection panel includes 73 Gram-positive bacteria, 12 Gram-negative bacteria, 6 fungi and 3 drug resistance markers (*vanA, vanB* and *mecA*). Sample preparation for the test involves selective lysis of human cells to increase detection of pathogen nucleic acids. The Magicplex assay uses dual priming oligonucleotides (DPO) for nucleic acid amplification. DPOs consist of two priming segments separated by a linker - a longer 5'-segment that initiates stable priming, and a short 3'-segment that determines target-specific extension. The design of DPOs make them highly specific [57]. The DPO primers are fluorescence labelled to allow real-time detection of the amplicon. The Magicplex assay can be completed within 6hrs [58].

In a study to assess clinical utility of the assay, 267 patients from the intensive care and emergency departments were enrolled of which 98 (37 %) specimens were positive. After clinical assessment, 63 (64%) positive specimens were considered BSIs (the remainder were considered contaminants) of which 23 (36%) were positive by both the Magicplex and blood culture, 22 (35%) were positive only by blood culture, and 18 (29%) were positive only by the Magicplex [58]. Sensitivity and specificity were 65 % and 92 %, respectively, for the Magicplex test and 71 % and 88 %, respectively for blood culture. The authors conclude that redesign is required to improve sensitivity and to simplify the procedure [58].

Additional tests are available for the molecular diagnosis of specific pathogens that can be used in blood such as the GeneXpert MRSA (Cepheid) or StaphPlex (Qiagen) tests, however, we have focussed our review on recent tests designed specifically for the diagnosis of BSI/sepsis.

***Limitations of molecular blood testing methods***

The true clinical impact (i.e. number of patients whose antibiotic treatment was adjusted based on molecular test results) of many of the described tests has not been reported in many of the studies. More comprehensive clinical diagnostic trials of these tests are required before their impact can be properly assessed. As mentioned previously, the clinical utility of blood culture based molecular tests is questionable due to competing technologies, test panel limitations and turnaround time. Culture independent molecular assays, on the other hand, can rapidly identify pathogens in whole blood compared to conventional blood culture and are more likely to have a clinical impact in future. Nonetheless, as expected, the clinical utility of the current assays is limited because of the predefined pathogen detection panels used in some tests and the limited/lack of information provided on antimicrobial susceptibility (Table 2) [59]. Furthermore, studies have shown clinically significant bacteraemia in adults is characterised by low pathogen numbers e.g. 10 CFU/ml in 73% of adults with gram negative bacteraemia and 1 to 30 CFU in more than half of adults patients with Staphylococcal and Streptococcal endocarditis [60]. Assays like SeptiTest® for example (detection limit 20-460 CFU/ml) may not be sensitive enough in such samples [54]. The rapid turn-around time, increased sensitivity and specificity (in some instances), capability to detect dead or viable but non-culturable pathogens in antibiotic pre-treated patient samples adds diagnostic value and has impacted therapy decisions in patients with suspected sepsis [46,61]. However, because of current limitations, these assays are used to complement blood culture rather than replace it [47].

Evaluation of molecular assays for the diagnosis of BSI is challenging due to the limited validity of the current ‘gold standard’ blood culture reference methods [56]. Although culture can detect 1 CFU of viable microorganism per 10 ml blood, the methods have low sensitivity for slow growing, intra-cellular and fastidious microorganisms and in antimicrobial pre-treated patients [18]. There have been improvements made in blood culture technology, such as development of new media, growth supplements and adsorbing reagents and the introduction of fully automated instruments [18,23]. Despite technology advancement and implementation of standard operation procedures, the overall positivity of blood culture can be as low as 30% to 40% [18]. Furthermore, time to positivity of blood culture is not fixed, it depends on growth rate of organism, time from sampling to incubation, volume of blood and initial CFU count [18]. Therefore culture methods are not ideal for evaluation of molecular techniques.

Some molecular methods are prone to contamination but more recent enclosed sample-to-result systems are far less likely to suffer from these problems. Any diagnostic method is only as good as the sample presented to it, therefore contamination of blood samples with microorganisms (e.g. from the skin) will be a problem for any diagnostic test. Contamination of the sample with microorganism nucleic acids (e.g. from the tube the blood is collected in or from the laboratory when the sample is being prepared for analysis), however, is a problem only for molecular methods. Better sampling, better consumables and more stringent laboratory protocol are required to ensure diagnostic accuracy. However, most of the molecular methods presented here have high specificity, suggesting that contamination is not a very big problem.

**Sequencing**

Sequencing technologies [e.g. MicroSEQ® 500 Kit (Perkin-Elmer Applied Biosystems, CA, USA) [62] and Pyrosequencing (Biotage, Sweden) [63]] have been used to identifying bacteria in positive blood cultures and directly from whole blood (SepsiTest®) using 16S/18S rDNA PCR amplicons. These sequencing technologies have been relatively cheap and rapid compared to conventional Sanger sequencing, however, their use has not provided expected diagnostic advantages [59]. They are hampered by contaminants, slow turn-around time and cost compared to conventional methods [59]. Additionally, because of the highly conserved nature of the 16S rRNA gene, identifying beyond genus level is sometimes impossible [64]. The revolution in bench-top next generation sequencing (NGS) technologies (e.g. Illumina MiSeq and Life Technologies Ion Proton) have made genome sequencing faster and cheaper and there is growing interest in applying these technologies for routine clinical microbiology [22].

***Turnaround time and cost effectiveness of molecular assays for the diagnosis of BSI***

We have reported the turnaround time of all the tests discussed (Tables 1 and 2), however this is not the total time required from when the blood sample was taken from the patient to the clinician receiving the result. This may be a more informative measure of turnaround time but it was not possible to provide this data as it was not reported in the original studies. The hospital specific systems in place for molecular blood testing e.g. samples tested immediately vs batch testing; local vs central testing; transport issues etc. mean that the molecular test used accounts for only a portion of the total turnaround time. The total turnaround time is something that should be considered when deciding to implement molecular tests for BSI diagnosis and efforts should be made to streamline the associated logistics, otherwise the benefit of rapid testing can be lost.

Molecular diagnostic tests are expensive (up to €300 per test [59]) compared to traditional culture reagents but are generally less laborious than culture and can shorten the turnaround time to results significantly. Few studies have been performed to evaluate the cost effectiveness of molecular assays for the diagnosis of BSI, with Septi*Fast* being the most studied. A study by Lehman et al concluded that for ICU patients with >25% incidence of inadequate empiric antimicrobial treatment, and at least 15% with a positive blood culture, the Septi*Fast* test represented a cost-neutral adjunct method [65]. In a more recent cost effectiveness study by Alvarez et al use of the Septi*Fast* test provided a significant economic saving. Mean total costs of clinical care were €42,198 in the control vs. €32,228 in the group in whom the Septi*Fast* test was used [66]. The mortality rate was similar in both groups, but the additional use of Septi*Fast* results shortened the ICU stay and lowered the use of antibiotics, which resulted in the reported saving.

A Verigene BC-GP test study reported the mean hospital costs of patients whose care included the use of BC-GP were $60,729 less than patients diagnosed traditionally. The authors postulate that the savings could be attributed to early initiation of or switching to appropriate antimicrobial therapy in response to BC-GP test results as well as ancillary recommendations on disease state management, including repeat blood cultures, echocardiograms, and infectious disease consultations [67].

**Five-year view and expert commentary**

Molecular methods based on seeking specific targets for pathogen identification and resistance genes are likely to be phased out in the coming years and replaced by more comprehensive methods such as mass spectrometry and genomic sequences methods [68]. Using next generation sequencing (NGS) to identify pathogens directly from clinical samples could enable the identification and genotyping of viable, dead and viable but non culturable bacteria, fungi and viruses there as well as detecting antibiotic resistance markers, virulence determinants and novel pathogens [68,69]. A study by Hasman *et al* demonstrated the utility of NGS for infectious diseases diagnostics by sequencing urine samples from patients with suspected urinary tract infections - pathogens and resistance markers were identified within 24 hours [70]. Seth-Smith and colleagues have also used NGS to sequence *Chlamydia trachomatis* directly from a clinical sample [71]. NGS is now commonly used in research and in reference laboratories for outbreak investigations such as *E. coli* O104:H4 [72], *S. aureus* and *Clostridium diffi­cile* [73] and *Salmonella* Typhi [74]. Implementation of NGS technologies in clinical microbiology for diagnosis of BSI is faced with a number of challenges. One challenge is development of accurate sequencing platforms that can provide clinically useful information faster than blood culture methods [68,75,76]. Another challenge in deploying NGS in routine diagnostics is the development of bioinformatics tools that will identify clinically relevant sequence information and communicate the results to microbiologists and clinicians in a timely fashion [64]. But perhaps the biggest challenge to applying NGS to BSI diagnostics is the detection of tiny amounts of pathogen nucleic acid in the vast excess of human nucleic acid present (ratio of approximately 1010:1 human to pathogen DNA per mL blood). Getting sufficient pathogen sequence from blood to be clinically useful would require terabases of sequence data, which clearly isn’t feasible. The solution to this problem is pathogen DNA enrichment – blood samples will need to be prepared to capture pathogen DNA (or cells) or remove as much human DNA (or cells) as possible while still providing sufficient pathogen DNA for NGS library preparation and sequencing.

Sooner rather than later NGS will be routinely applied in clinical microbiology laboratories - Illumina and Pacific Biosciences are some of the big players interested in this area [39]. Oxford Nanopore Techologies have developed a new generation of nanopore-based single molecules sequencing. Their MinION™ device is the size of a large USB stick and is designed to provide real-time data streaming and improved simplicity. This data streaming feature allows rapid (within minutes) pathogen identification and the MinION™ device demonstrates great potential for application in infectious diseases diagnostics and epidemiology [74] as well as other areas [39]. Currently none of the NGS technologies have been approved for infectious diseases diagnostics, however more research and technological development will fast track NGS into routine use in clinical microbiology [39].

As described earlier, MALDI-TOF MS is now used for routine microbiology analysis and more labs are expected adopt this technology for the rapid, cost-effective and comprehensive identification of bacteria in positive blood culture. Mass spectrometry is likely to also be used for the analysis of pathogen DNA directly from blood samples in the near future. PCR electrospray ionization-mass spectrometry (PCR/ESI-MS) may give similar benefits as MALDI-TOF [77]. The Iridica system (Abbott, USA) and its predecessor technology PLEX ID® are designed for pathogen detection in biological samples. The platforms are based on broad range PCR amplification of a number of target genes followed by analysis of PCR amplicons using ESI-MS [78,79]. ESI-MS signals are translated into amplicon base composition signatures to provide a fingerprint of the organism/s in the sample [79]. The Plex-ID® system was taken off the market by Abbott and has now been replaced by the next generation Iridica system (CE marked as of December 2014). According to the manufacturers, their bloodstream panel identifies 780 bacterial strains (a fungal panel is also available) and four antibiotic resistance markers allowing unbiased screening of patient samples in less than six hours. Abbott are targeting the critical care market with this technology and one of the major applications is for BSI/sepsis diagnosis.

There are some technologies on the horizon that may have an impact on the diagnosis of BSI/sepsis in the near future. DNA Electronics Ltd (DNAe) is developing an integrated instrument called Genalysis®, which is based on PCR/semiconductor sequencing technology. The first diagnostic product for this system will be a test for the rapid diagnosis of BSI, which will identify pathogens and antimicrobial resistance genes directly in whole blood in 2-3 hours. DNAe have recently acquired nanoMR and will integrate their sample preparation technology, immunomagnetic pathogen capture system (PCS), into the Genalysis system. The PCS can target multiple bacteria and fungi in the blood with a manufacturer reported sensitivity of 1 cell/mL in less than 30 minutes.

GeneWEAVE are developing the vivoDx system for the diagnosis and antimicrobial susceptibility testing of pathogens in clinical samples including positive blood culture. The system is based on Smarticles technology which combines DNA-delivery bio-particles with DNA that causes the bacteria to express luciferase. Samples can be tested in the presence of antibiotics to provide a molecular based antimicrobial susceptibility test that can help guide therapy. The turnaround time is expected to be less than 4 hours.

**Conclusions**

Molecular methods for pathogen identification from blood culture or directly form whole blood have not been widely implemented due to sub-optimal accuracy caused by the limited range of pathogens and resistances they can detect. Technologies such as MALDI-TOF and PCR/ESI-MS are capable of detecting broader ranges of pathogens and are likely to be the methods of choice in the coming years for pathogen identification in blood culture and directly from whole blood, respectively. However, these methods are hampered by their limited ability to test for antimicrobial resistance. Studies have shown that NGS has the potential to be used for the routine clinical diagnosis of infectious diseases. Further development of sequencing platforms, sample preparation methods and bioinformatics tools will see this vision realised in the near future.

**Key issues**

* The poor sensitivity and long turnaround time of blood culture, the ‘gold standard’ diagnostic for BSI/sepsis, results in inappropriate antibiotic prescribing and increased patient morbidity and mortality.
* Rapid and comprehensive molecular diagnostics have the potential change clinical prescribing and improve clinical outcomes and antibiotic stewardship. However, current methods are limited by the range of pathogens and resistances they can detect.
* Molecular diagnostics can be used on positive blood cultures or directly on patient blood samples. The implementation of MALDI-TOF MS in clinical microbiology laboratories is likely to replace molecular methods for pathogen identification in blood culture. Hence, molecular methods for direct diagnosis in whole blood are likely to be more important in future but current methods lack the comprehensiveness to replace culture.
* NGS based diagnostic methods have the potential to overcome the shortcomings of PCR-based technologies by offering a comprehensive approach to rapid pathogen and antibiotic resistance marker detection in blood. Rapid pathogen DNA enrichment strategies will be required for this approach to become feasible.
* Blood culture is not sufficiently sensitive to be considered as a ‘gold standard’ for the diagnosis of BSI/sepsis and is unsuitable for determining the performance of new diagnostics methods.

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Table 1. Commercially available methods for pathogen detection in positive blood culture

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Assay | Technology | Pathogen panel | Antimicrobial resistance genes | Sensitivity | Specificity | Turnaround time (h) | Reference |
| Prove it™ sepsis  (Mobidiag® Finland) | Multiplex PCR + microarray | 60 Bacteria +13 fungi targets | mecA, vanA/B | 96-99% | 98-100% | 3hrs | [25,26,80] |
| FilmArray® (BioFire Diagnostics, USA) | Real-time multiplex PCR | 24 species/genus +5 fungi targets | *mecA, vanA/B* and KPC | 80.4-94.3% | 95-100% | 1hr | [37,81,82] |
| Verigene®(Nanosphere Technology, USA) gram-negative bacteria (BC-GN) | Hybridization on microarray | 9 species/genus gram negatives targets | CTX-M (ESBL) and 5 markers for carbapenemases | 89-100% | 93-100% | 2.5hrs | [31,83,84] |
| Verigene®(Nanosphere Technology,USA) gram-positive bacteria (BC-GP) | Hybridization on microarray | 13 species/genus bacterial targets | *mec*A, *van*A, and *van*B | 92.6-100% | 95.4-100% | 2.5hrs | [30,34,85,86] |

Table 2. Commercially available methods for direct pathogen detection in the whole blood

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Assay | Technology | Pathogen panel | Detection limit (cfu/ml) | Sensitivity | Specificity | Turnaround time (h) | Reference |
| T2Candida® test(T2Biosystems inc) | PCR + magnetic resonance technology | 5 Candida species | 1 | 100% | 98% | 3hrs | [43] |
| SeptiFast (Roche Diagnostics, Germany) | Real-time PCR | 25 Species/genus targets of bacteria and fungi | 3 to 30 | 68-75% | 86-92% | 6hrs | [51,52] |
| SepsiTest (Molzym, Germany) | PCR + sequencing | 345 Bacteria + fungi | 20-460 | 86-87% | 83-85% | 8 to 12 hrs | [54,87] |
| Vyoo® (SIRS, Germany) | PCR + electrophoresis | 34 Bacteria, 6 fungi targets + 5 resistance markers | 5 to 100 | 60% | 70-75% | 7hrs | [55,88] |
| Magicplex™  (Seegene, Korea) | Real-time PCR | 85 Bacteria  6 fungi + 3 resistance markers | - | 65% | 92% | 6hrs | [58] |