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Blood Culture-Based Diagnosis of Bacteremia: State of the Art.

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Abstract

Blood culture remains the best approach to identify the incriminated microorganisms when a bloodstream infection (BSI) is suspected, and to guarantee that the antimicrobial treatment is adequate. Major improvements have been made in the last years to increase the sensitivity and the specificity and to reduce the time to identification of microorganisms recovered from blood cultures. Among others, the introduction in clinical microbiology laboratories of the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) technology revolutionized the identification of microorganisms whereas the introduction of nucleic-acid based methods such as DNA hybridization or POCT-PCR significantly reduce the time to results. Together with traditional antibiotic susceptibility testing (AST), new rapid methods for the detection of resistance mechanisms respond to major epidemiological concern such as MRSA, ESBL or carbapenemases.

Introduction

Bloodstream infections (BSIs) are severe diseases characterized by a high morbidity and mortality, which is directly related with the delay in the administration of the first adequate anti-infectious agent [1-7]. Empirical anti-infectious treatments are chosen on the basis of the clinical and epidemiological data and are started immediately after the sampling of the blood vials; however until microbiological documentation, their adequacy cannot be guaranteed [8-10]. Microbiological investigations – identification of the causative agent and antibiotic susceptibility test (AST) - are thus very important: 1) to adjust the anti-infectious therapy and to avoid inefficient treatment, 2) to reduce the spectrum of the anti-infectious therapy in order to limit the selection of resistant strains and 3) to limit the toxicity and negative impact on beneficial bacteria of some broad spectrum molecules or combined therapy. In adults the quantity of microbes present in the blood during BSIs ranges from 1 to 10 cfu per ml [11-14] to $1x10^3$ and $1x10^4$ [15]. In children the bacterial load during BSI might be higher. Blood cultures currently represent the main method to determine the etiology of a BSI because they are highly sensitive and easy to perform. The sensitivity of blood culture is largely due to the volume of the sample. For adults one blood sampling generally represent up to 20 ml of blood used to inoculate 2 vials (one aerobic vial and one anaerobic vial). Before antibacterial treatment 2 to 4 blood cultures, i.e. 40 ml to 80 ml of blood are necessary to detect a causative agent in 80% to 96% of bacteremia [16, 17]. In clinical practice however, the sampling is often limited to 2 blood cultures. Nevertheless, approximately 50 % of the BSI remains blood culture negative [18, 19] and further improvements may be foreseen.

Blood culture sampling and technologies

Standard vials containing rich media have been designed for aerobic and anaerobic growth conditions, respectively. They are dedicated for up to 10ml ml of blood. However, because of the difficulty to obtain large volume of blood, specific pediatric blood vials have been designed for the culture of volume lower

than 3ml. To neutralize antibiotics given prior sampling charcoal or resins have been introduced in specific vials. Lytic agents added in some growth media promote the recovery and growth of organisms that have been endocytosed by phagocytes. Standard incubation time is around 5-7 days which is sufficient for the recovery of the majority of organisms including HACEK bacteria and *Brucella* spp. [20, 21]. However, the incubation time should be increased for slow growing organisms such as fungi and *Mycobacteria* spp.; the latter being grown in vials containing Middlebrook 7H9. Modern laboratories rely on automated incubators (Table 1) including continuous monitoring for positive vials detection which significantly reduces the incubation time and the contamination rate. Blood culture positivity is generally detected by following CO_2 production by growing microorganisms that trigger a pH increase visualised by colour changes, fluorescence signal or red-ox variations.

Interpretation of positive blood-cultures: contamination *versus* bloodstream infection

Contamination that represents up to 1/3 of positive blood cultures can occur when microbes not present in the bloodstream are introduced in the vial during the blood sampling [22]. Even if blood cultures are drawn under aseptic conditions, contaminations are often due to organisms of the skin flora such as coagulase negative staphylococci (CoNS), the 3rd most prevalent microbe identified in positive blood cultures (Figure 1), or to organisms from the environment with low or absence of virulence towards humans such as *Micrococcus* spp., *Propionibacterium acnes*, most *Bacillus* spp. and most *Corynebacterium* spp. [22]. Moreover, an inadequate blood volume increases the rate of contamination. Interestingly, peripheral venipuncture, arterial access or central venous accesses are associated with different contamination rates of 36%, 10% and 7% respectively [22]. For venipunture a promising approach to reduce to less than 30% the contamination rate is named the initial specimen diversion technique (ISDT), in which the first blood milliliter potentially containing bacteria not killed by skin surface antisepsis is discarded or used for other purposes [23, 24]. Nevertheless the specificity of blood culture for the diagnosis of BSI remains high as

most of the top microbes recovered from positive blood cultures are primarily pathogens such as *Escherichia coli*, *Staphylococcus aureus* or *Pseudomonas aeruginosa* respectively 2nd, 4th and 5th position (Table 1).

Together with the clinical signs and symptoms, several parameters can help to precise the significance of positive blood cultures: 1) the number of positive vials, the number of positive blood culture pairs as well as the proportion of positive cultures [25], 2) the site of sampling - catheter *versus* venous puncture - and 3) the time to positivity, including the differential time to positivity between pairs collected from different sampling sites [26]. For this reason, international guidelines recommend drawing several set of blood vials [27]. Increasing the number of blood cultures increases the sensitivity due to the increased total volume collected [28]. Blood culture sampling should be repeated since bacteremia often occurs in intermittence. This is not the case of endocarditis or septic thromboplhebitis, where all the bottles are generally positive with pyogenic bacteria such as *S. aureus* and *S. pyogenes*.

When contamination is excluded, the organism detected and identified in the blood culture is likely present in the bloodstream at the time of sampling, which defines a bacteremia (or fungemia). Bacteremia or fungemia can be transient or sustained. Transient bacteremia correspond to a single episode of positive blood cultures due to the presence of microorganism during a short time-lapse in the bloodstream (less than 30 min). They are generally caused by the manipulation of contaminated mucosa or invasive respiratory, gastro-intestinal or urogenital acts [29]. Conversely, multiple positive vials drawn at different time is synonymous of sustained bacteremia (fungemia). Sustained positive blood cultures are encountered in presence of endovascular infections such as endocarditis. In such cases a high number of blood cultures become positive without any difference in the time to positivity for bottles drawn simultaneously from different sites. In contrast a catheter infection is suspected when blood cultures drawn from a catheter become positive more than 2 hours before blood culture drawn from a venipuncture. This time to positivity difference exhibits an overall sensitivity of 91% and a specificity of 94% for the diagnosis of catheter infection [30]. The catheter can be removed and cultured by plating the catheter tip using the Maki roll-on semi-quantitative method [31]. Sustained bacteremia should be differentiated from persistent bacteremia

that is defined by the persistence of positive blood cultures despite the introduction of an anti-infectious treatment. Persistent bacteremia (fungemia) are generally due to organisms resistant to the prescribed antibiotic, to the presence of a second organism or to antibiotic inaccessibility to the site of infection (e.g. septic thrombosis).

Identification and antibiotic susceptibility testing from positive blood culture

Upon blood culture positivity, the first step is to perform a Gram staining with a blood culture aliquot. This is a mandatory analysis to confirm the presence of bacteria/fungi in the blood vial. If microbes are present, the morphotype provides a first hint on the etiology of the infection. Then microbes identification can be achieved: 1) starting from a subculture, 2) directly from the positive blood culture using nucleic acid-based methods such as hybridization and microarray or, 3) after a bacterial enrichment and purification step to obtain a “bacterial pellet”, suitable for a variety of approaches including matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Figure 2).

Identification approaches requiring a pure subculture

After the Gram staining examination, the positive blood culture can be plated to insure pure culture (isolated colonies) that can be analyzed using conventional biochemical methods, MALDI-TOF MS and/or nucleic-acid based methods such as PCR and gene sequencing (Figure 2). A 12 to 24h incubation period is generally required to obtain enough isolated colonies for biochemical characterization whereas relatively short incubation (<6 hours) are often long enough for MALDI-TOF MS identification, the current best method for rapid bacterial identification. AST performed directly from a positive blood vial without a pellet preparation or a subculture is not recommended. Different AST methods can be applied from isolated colonies. Automated methods such as Vitek (BioMerieux) allow the testing of a high number of molecules at the same time. The interpretation of the result is automated and internal expert systems are able to detect

specific resistance mechanisms such as ESBL and carbapenemases. However, they do not give an exact MIC in contrast to E-test.

Nucleic-acid based methods that can be used directly on positive blood cultures

Molecular methods are generally faster than phenotypic approaches because they are culture independent. However, the use amplification-based nucleic acid methods such as PCR have to face several limitations associated with a blood sample: 1) presence of PCR inhibitors 2) presence of a high quantity of non-microbial nucleic acids 3) presence of contaminant DNA and 4) persistence of DNA from dead microbes (Opota et al 2015 on the same issue). Probes hybridization and microarrays that are non amplification-based nucleic acid methods are less influenced by inhibitors and less prone to contamination but they require a high bacterial or fungal load. Hence these methods are used downstream positive blood cultures in which the bacterial load can reaches 1.10^6 to 2.10^8 for Gram-positive cocci and 2.10^7 to 1.10^9 for Gram-negative bacilli (Figure 2) [32, 33] (Opota et al 2015 on the same issue).

Fluorescent in situ hybridization (FISH) that consists in the specific binding of fluorescent nucleic acid probes on complementary pathogens DNA sequences - 16S rRNA for bacteria and 18S rRNA for fungi - can be performed directly from the positive blood culture. The specific binding is observed using a fluorescent microscope. The choice of the probe is dependent on the Gram staining: staphylococci probes (*S. aureus*/CoNS), enterococci probes (*E. faecalis*/*E. faecium*), Gram negative probes (*E. coli*/*P. aeruginosa*/*K. pneumoniae*), yeast probes (C. *albicans*/C. *glabrata*/*C. parapsilosis*/*C. krusei*) for instance. The commercial solutions PNA-FISH and Quick-FISH (AdvanDx, USA) display a time to result of about 1.5 to 3 hours with a sensitivity and specificity of 97-100 % and 90-100 % respectively (Table 2)[34-39]. The AccuProbe system (Gen-Probe, San Diego, California) is based on DNA probe that can detect S aureus, Streptococcus pneumoniae, Enterococci spp., and group A and B streptococci . The sensistivity and specificity are above 97% at the exception of the *S. aureus* probes that demonstrate a sensitivity of 99.8% and a specificity of 80.8% [40, 41].

Microarrays allow the detection of a limited number of species which cover 90 to 95% of all the pathogens causing BSI. The turn-around time is about 2.5 to 4 hours with sensitivity ranging from 10 to 10^5 CFU/ml [42]. In contrast to FISH, microarrays generally contain probes for the detection of resistance genes such as *mecA*, *vanA*/*vanB* and bla_{KPC}. For example, the Verigene system (Nanosphere, Northbrook, IL, USA) consists in two distinct kits for the detection of 12 Gram positive and 9 Gram negative bacterial species with a sensitivity ranging from 81 to 100% and a specificity higher than 98% [43, 44]. The Prove-it Sepsis assay (Mobidiag, Finland) combine a PCR with a microarray for a turnaround time of 3.5 hours, 95% of sensitivity and 99% of specificity (Table 2) [45].

POCT-PCR systems that allow the detection and identification of methicillin sensitive *Staphylococcus aureus* (MSSA) and methicillin resistant *Staphylococcus aureus* (MRSA) have been dedicated to blood cultures because of the clinical impact and the epidemiological concern of this pathogen [46, 47]. MSSA/MRSA detection POCT such as, GeneXpert MRSA/SA BC Assay (Cepheid, Sunnyvale, CA) [48, 49] and the StaphSR assay (BD GeneOhm, San Diego, CA) [50, 51] are based on multiplex real-time PCR to detect *S. aureus* and the presence of the *mecA* gene. The results are obtained in approximately 1.5 h. The FilmArray (Idaho Technology, Salt Lake City, UT, USA) is a multiplex PCR-based system designed to detect 25 microbes (90 to 95% of the pathogens involved in blood cultures) and the resistance genes *mecA*, *vanA* and *vanB* and bla_{KPC} [52].

Subculture independent approaches: identification and antibiotic susceptibility testing using a blood-culture microbial pellet.

The use of matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) technology for the identification of microorganisms is among the major revolution in clinical microbiology laboratory of the last years. Microbial identification using MALDI-TOF MS is based on the comparison of a protein profile obtained by mass-spectrometry from a bacterial or fungal sample with a database of profile obtained from characterized microbes [53, 54]. MALDI-TOF MS is an accurate and fast approach, which

performance largely depends on microorganism's purity and quantity. Therefore, bacterial enrichment and purification procedures are required from positive blood cultures which contain high concentration of non microbial material that may interfere with MALDI-TOF MS identification and AST. Several in-house and commercial methods have been developed to isolate and concentrate microorganisms from positive blood cultures including lysis centrifugation methods [55-57], saponin or equivalent mild detergent methods [58, 59], serum separator method [60] and the commercial MALDI Sepsityper Kit (Bruker Daltonics Inc., Billerica, MA) [61]. These pellet preparation protocols may not only be used for MALDI-TOF MS identification but also provide enough starting material for other downstream applications including Gram staining, AST [62] and POCT PCR [49].

MALDI-TOF MS on positive blood-culture pellet

The main MALDI-TOF MS systems commercially available, the Autoflex II mass spectrometer (Bruker Daltonik) and Axima Assurance system (Shimadzu Corporation) display similar performances [53, 54, 63, 64]. The performances of MALDI-TOF MS identification vary according to the enrichment and purification method (Table 2). In the study performed by Prod'hom et al., a 78.7% correct identification by MALDI-TOF MS was obtained from blood culture pellets. Among samples giving no reliable identification by MALDI-TOF MS, 81% were blood culture positive for gram positive bacteria including mainly streptococci and coagulase-negative staphylococci [55]. Using a differential centrifugation protocol, March-Rossello et al. correctly identified 97.3% of Gram negative bacteria and 98.4% of Gram-positive bacteria [65]. Poor MALDI-TOF MS identifications from blood culture preparation are mainly observed with difficult-to-lyse bacteria (e.g. *Klebsiella pneumoniae*), closely related bacterial species (e.g *Streptococcus mitis* group) and bacteria such as anaerobes that are poorly represented in the MALDI-TOF MS database [53].

Antibiotic susceptibility testing (AST) on positive blood-culture pellet

Automated microbial systems cards and manual disk diffusion assays were directly used to perform AST from purified and/or enriched microbial samples obtained from positive blood culture. Machen *et al.*

performed a same day AST by directly inoculating the AST automated microbial system (VITEK 2) with filtered microorganims from positive blood cultures. A 93.5 % category agreement with tested antibiotics was obtained with only 1.7 % major error (ME) and 1.3 % very major error (VME) according to definitions given by the FDA for interpretive agreement results [66]. Similar results were observed when the VITEK 2 was inoculated with bacterial pellets obtained by ammonium chloride lysis centrifugation [62] or when the BD Phoenix (BD Diagnostics) system was inoculated with bacteria harvested using serum separator tubes [67]. However, some antibiotics known to present frequent discrepancies compared to conventional approaches need to be confirmed by disk diffusion assays and/or E-test directly performed from the same blood culture bacterial preparations [62].

The emergence of broad spectrum antibiotic resistance mechanisms triggered the development of methods allowing rapid detection of ESBL and Carbapenemase activities on blood cultures positive for Gram negative bacteria. Using a Triton lysis-centrifugation method, ESBL activities can be directly and rapidly (less than 1 hour) detected from spiked blood culture using the ESBL NP test with 100% sensitivity and specificity [68]. Interestingly, the ESBL NP test applied on blood culture pellets showed a higher performance compared to the same test performed on bacteria grown on agar plates which exhibited a 100% specificity; the decreased sensitivity (92.6%) is due to the poor performance of the test (25% sensitivity) for the detection of non-CTX-M ESBLs producers. The higher performance observed with this test applied on blood culture samples is likely explained by the very high bacterial inoculum recovered from positive blood culture. Another approach to detect ESBLs from positive blood cultures is the chromogenic cephalosporin HMRZ-86 βLACTA test (BioRad) exhibiting a 100% sensitivity and specificity following a 2 hours subculture in tryptone soya broth (TSB) to prevent inhibition of the test by lysed blood [69]. Thus in Lausanne, we applied it on purified bacterial pellets with accurate results (Prod'hom *et al.* submitted). The detection of carbapenemase activity from positive blood cultures can be performed with the Carba NP with 97.9 % sensitivity and 100% specificity test following a 3 hours selective enrichment in brain-heart infusion (BHI) containing imipenem [70]. A lower detection sensitivity (91.3%) was observed with OXA-48 producers whereas 100% of sensitivity was obtained with other classes of carbapenemases.

POCT PCR on positive blood-culture pellet

Rapid PCR-based test such as the GeneXpert MRSA/SA BC Assay (Cepheid, Sunnyvale, CA) can be applied on both native and microbial purified and enriched fraction of positive blood cultures (Figure 2). The GeneXpert MRSA/SA test was applied on *Staphylococcus aureus* blood culture bacterial pellets identified by MALDI-TOF MS and showed 99% of sensitivity and 100% of specificity, allowing a significant reduction of anti-MRSA antibiotics misuse from 26.1% to 8.1% [49].

Conclusions

New technologies and new methods for the diagnostic of positive blood cultures allow a significant reduction of the TAT for both identification and AST, with a positive impact on the management of patients suffering from bloodstream infections.

Some of these approaches such as microbial enrichment via centrifugation require significant hands on time and experienced lab technician, which may hinder their implementation in laboratories that process large volumes of positive blood cultures and/or that have limited human resources. Thus, the emergence of new laboratory methodologies and new laboratory automated technologies, should help the implementation of these new diagnostic approaches.

The example of recent development for the diagnosis of bloodstream infections highlight the importance of a dynamic R&D process in diagnostic laboratories that promote innovation and implementation of the most recent technologies for the benefit of patients care, while keeping an acceptable cost per test ratio.

Transparency declaration

No conflict of interest declared.

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Figure legends

Figure 1: Top 20 of the microbes identified from positive BC during 1 year. Data from our 1000-bed tertiary care university hospital during the year 2013. The pie chart represents the distribution per morphotypes of all the microorganisms (total = 16682 identifications).

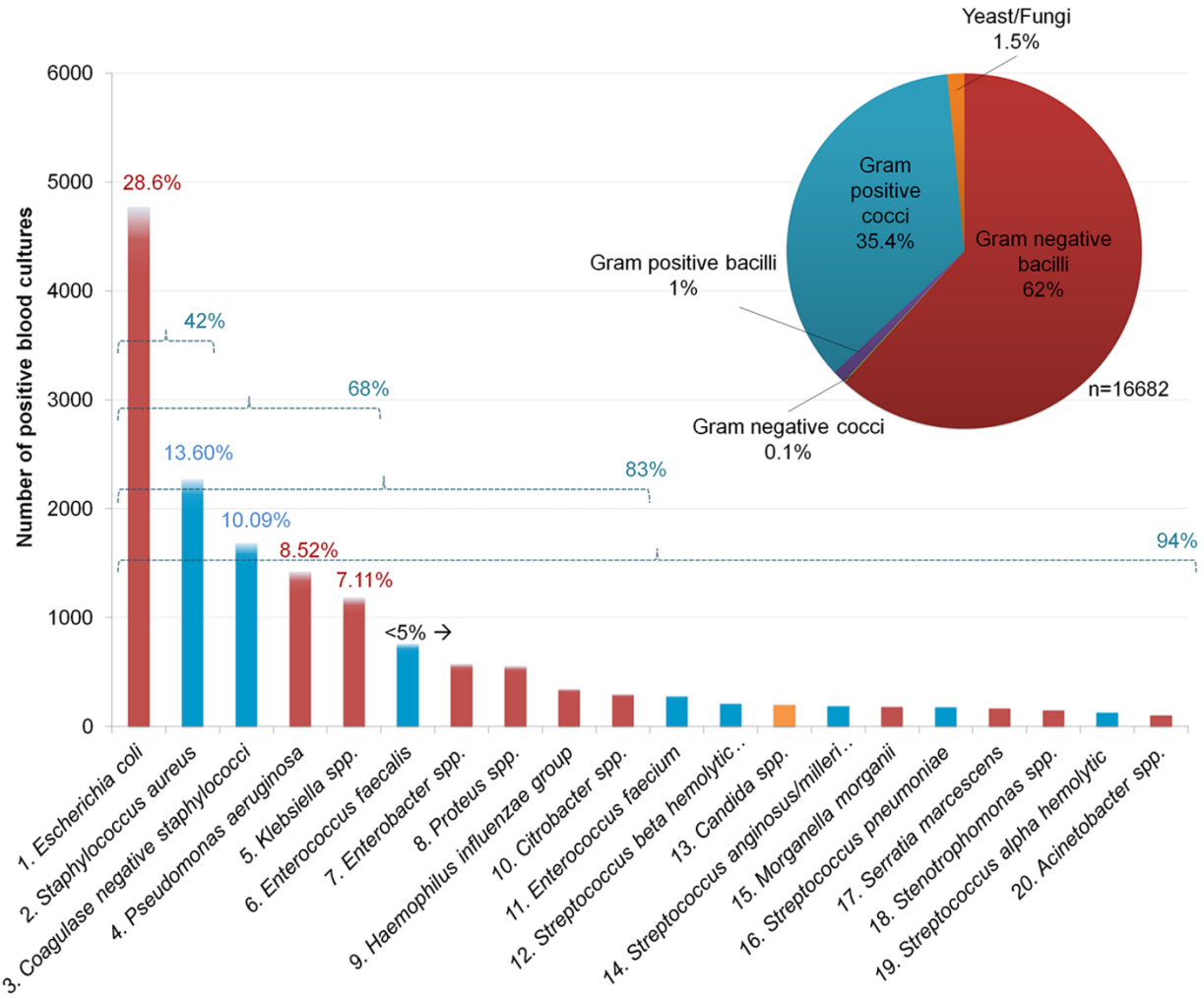
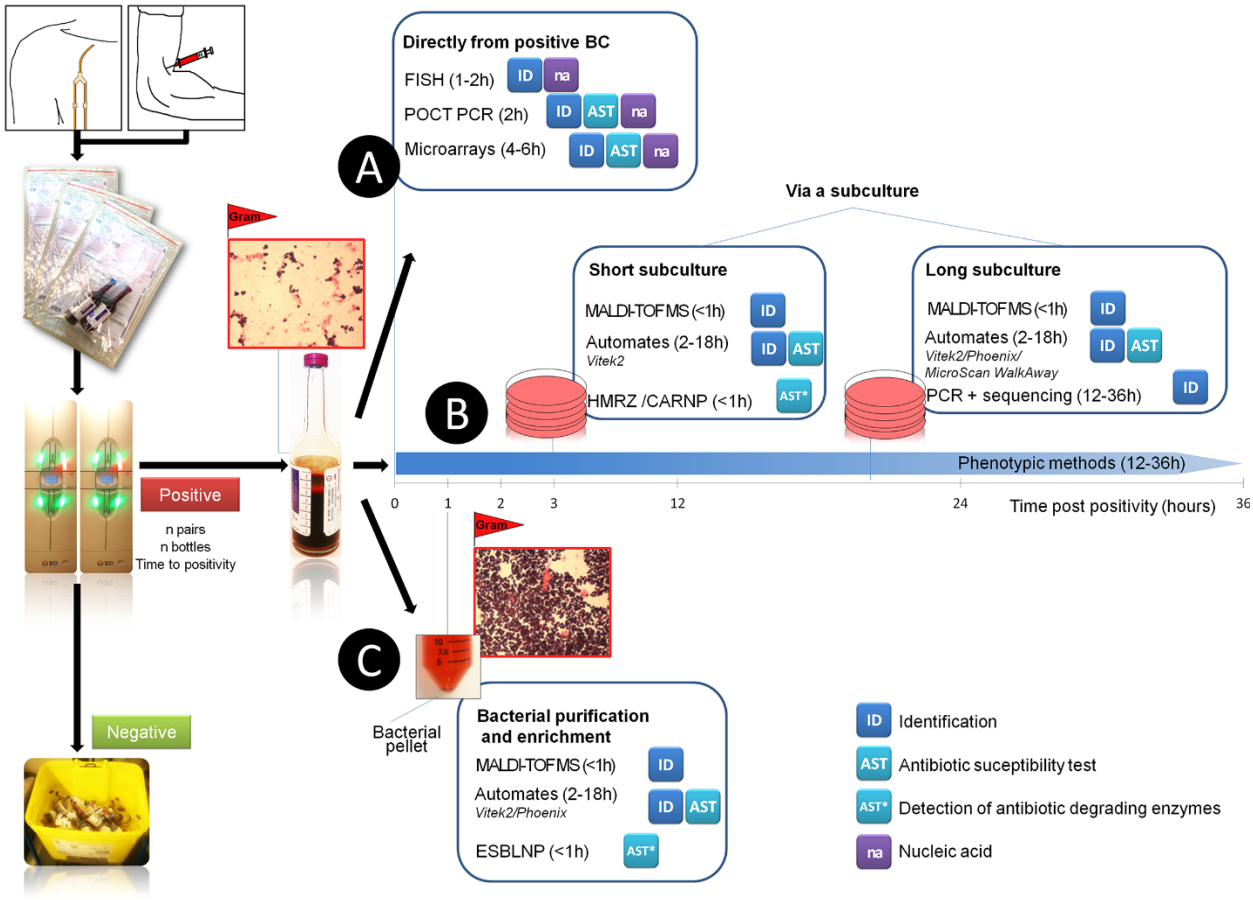


Figure 2: Methods to identify microorganisms from positive blood cultures. A) Directly from positive blood culture, B) via a subculture step and C) using a purified bacterial.



548 **Table 1:** Main automated blood culture incubation systems currently commercially available.

System (Manufacturer)	Main blood culture media and chartacteristics	Positivity detection system
BD BACTEC (BD Diagnostics, Franklin Lakes, NJ, USA)	- Standard aerobic and anaerobic broth media. - Media containing resin particles. -Media specifically designed for small blood volume inoculation. -Media containing a lysing agent to increase the recovery of organisms phagocytozed. -Media optimized for the growth of mycobacteria. - Specific algorithms for fastidious organisms (e.g *Haemophilus* spp and *Neisseria* spp.).	fluorescent sensor of CO2 production
BacT/ALERT 3D (bioMérieux, Durham, NC)	-Plastic bottles. -Standard aerobic and anaerobic broth media. -Media containing activated charcoal particles*. -Media specifically designed for small blood volume inoculation. -Media supplemented with Middlebrook 7H9 for micobacteria growth. -Enriched media.	colorimetric sensor of CO2 production
VersaTREK, (TREK Diagnostic Systems, ThermoFisher Scientific, Waltham, MA)	- Standard aerobic and anaerobic broth media for samples from 0.1 to10 ml, optimized to minimize the impact of antibiotics.	Monitoring of red-ox variations

* The presence of charcoal particles prevent the use of the pellet for direct idenfication from positive BC using MALDI-TOF MS

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551 **Table 2:** Characteristics of the commercially available systems for the identification of microbes from

552 positive blood culture

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System	Methods	Time to result	Microorganisms coverage	Resistance and virulence markers	Sensitivity Specificity Correlation with conventional methods (%)	Comments	References
PNA FISH and QuickFISH (AdvanDx, Wolburn, MA)	FISH	<1-3 hours	4 Gram positive 4 Gram negative 5 Fungi	0	97-100 90-100 96-99	+ : rapid, sensitive and specific - : dependent on the choice of the probes to be tested, no resistance marker	[34, 37, 38, 71-76]
AccuProbe (Gen-Probe, San Diego, CA, USA)	FISH	<1 hour	*S. aureus* *Enterococcus* spp. *S. sneumoniae* *Streptococcus* group A *Streptococcus* group B	0	80.8-100 98.7-100 nr	+ : high specificity - : variable sensitivity, no resistance marker, limited number of publications	[40, 41]
Verigene (Nanosphere, Northbrook, IL, USA)	Microarray	2.5 hours	12 Gram positive 9 Gram negative	*mecA*, *van*A/B, KPC, NDM, CTX-M,VIM,IMP,OXA12	81-100 98-100 nr	+ : detection of resistance markers, good specificity - : variable sensitivity, narrow range of pathogens detected	[43, 77-85]
Prove-it Sepsis (Mobidiag, Finland)	Microarray	3.5 hours	60 bacteria 13 fungi	*mecA*	95 % 99 % nr	+ : sensitive and specific - : limited number of publications	[45]
FilmArray (Idaho Technology, Salt Lake City, UT, USA)	Multiplex PCR	1 hour	8 Gram positive 11 Gram negative 5 Fungi	*mecA*, *van*A/B, KPC	97-95 91-98 nr	+ : rapid, sensitive and specific - : narrow range of pathogens detected	[52, 86-88]
Xpert MRSA/SA BC (Cepheid, Sunnyvale, CA, USA)	Real-time PCR	1 hour	S. aureus	*mecA*	100 99-100 nr	+ : rapid, sensitive and specific - : expensive	[48, 49, 89-91]
StaphSR assay (BD GeneOhm, San Diego, CA, USA)	Multiplex PCR	1-2 hours	S. aureus	*mecA*	96-100 95-98 nr	+ : rapid, sensitive and specific - : expensive	[92, 93]
StaphPlex (Genaco Biomedical Products, Huntsville, AL, USA)	Multiplex PCR + Microarray	5 hours	S. aureus	*mecA* (+ PVL)	100 95-100 92	- : limited number of publications	[94]
MALDI-TOF MS Brucker Daltonics (Bremen, Germany) bioMérieux (Marcy l'Etoile, France)	Mass-spectrometry	<1 hour	<1000*	not in routine	- - 76-99	+ : rapid, sensitive and specific - : significant hands on time for bacterial enrichement	[32, 35, 53, 55, 56, 62, 65]
nr=non reported *dependant on the mass-spectrum database							

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