“Molecular and Mass Spectrometry Detection and Identification of Causative Agents of Bloodstream Infections”

Onya Opota[a], Katia Jaton[a], Guy Prod’hom[a] and Gilbert Greub[ab]

Institute of Microbiology[a] and Infectious Diseases Service[b], University of Lausanne and University Hospital Center, Lausanne, Switzerland

Corresponding author:

Gilbert Greub,

Institute of Microbiology,

University of Lausanne and University Hospital Center,

Bugnon 44, 1010 Lausanne,

Switzerland.

Phone +41 (0)21 314 4979

Fax +41 (0)21 314 4060

E-mail: Gilbert.Greub@chuv.ch

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ABSTRACT

Bloodstream infections (BSI) are severe diseases associated with a high morbidity and mortality, which increases with the delay until the administration of the first appropriate antibiotic. For this reason, empirical treatments, made of broad spectrum antibiotics, are rapidly started when a BSI is suspected on the basis of the clinical and epidemiological data, but this does not exclude any risk of inappropriate initial treatment and make the microbial diagnosis crucial. The time to positivity of blood culture, currently the gold standard to establish the etiology of bloodstream infection can vary from few hours to several days and using conventional (culture-based) methods, one or more days can be necessary to determine the etiologic agent of a BSI. Recent advance in molecular biology have permit to develop new methods to accelerate the microbial diagnosis of BSI. Non-amplified nucleic acid-based methods such as fluorescent in situ hybridization (FISH) or microarray can be used on positive blood culture whereas amplified nucleic acid-based methods such as (real-time) PCR or PCR-Electrospray Ionization Mass Spectrometry (PCR/ESI-MS) can be used directly on whole blood. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS), based on the analysis of the mass spectrum generated by bacterial proteins can be used on positive blood culture. We will present these technologies and their performance; we will also discuss their advantages and their inconvenient as well as the question that need to be addressed to fully benefit from these new technologies.

INTRODUCTION

1. Bloodstream infections

Bloodstream infections (BSI) are severe diseases associated with a high morbidity and mortality, which increases with the delay until the administration of the first appropriate antibiotic (58; 82; 90; 113; 148; 149; 157; 232). For this reason, empiric treatments made of broad range anti-infectious compounds or made of a combination of antimicrobials are started immediately after the sampling of blood bottles. BSIs can be caused by various microorganisms. In the absence of microbiological documentation, a BSI is suspected by the physicians on the basis of clinical symptoms, which trigger the start of empirical treatments. The clinical presentations are multiple and include: fever or hypothermia, increases in heart rate, change in inflammatory variable (C-reactive protein, procalcitonin and white blood cell count increase), organ failure (58; 178). These symptoms are generally non specific and only suggest bloodstream dissemination. Empirical treatments are made of broad spectrum antibiotics on the basis of the clinical and epidemiological data, but this does not exclude any risk of inappropriate initial treatment. Similar to delayed introduction of the first antibiotic treatment, an inadequate treatment is associated with a significant increase of mortality (188; 265). In a recent study there was a nearly 3-fold increase in the risk of mortality when the antimicrobial treatment was inappropriate (28). *Pseudomonas aeruginosa* or *Acinetobacter baumannii* that are associated with frequent multi-drug resistant profile are often the microorganisms for which empirical antibiotic coverage are inappropriate. *Enterobacteriaceae* producing Extended Spectrum Beta-Lactamase (ESBL) such as *Escherichia coli* or producing inducible cephalosporinase such as *Enterobacter cloacae*, are also commonly associated with an inadequate empirical treatment (37). The mortality rate of patient suffering of a methicillin resistant *Staphylococcus aureus* (MRSA)-associated BSI was significantly higher among patients receiving an inappropriate empirical antibiotic treatment (168/342, 49.1%) than among patients receiving an appropriate empirical

antibiotic treatment (56/168, 33.3%) (92; 187). Inadequate treatments can also occur for organisms such as *Enterococcus faecalis* species that are intrinsically resistant to some commonly administered first-line antibiotics such as Ceftriaxone (265). Broad-spectrum molecules also have a detrimental impact on beneficial bacteria that constitute the protective flora and contribute to the emergence of multi-drug resistant strains. Moreover, some of these antimicrobial molecules can also have a toxic effect for the patient (144; 188; 265). The rapid identification of the causative agent of the BSI thus allows the adjustment of the anti-infectious therapy or the reduction of the spectrum (de-escalation) with significant clinical benefits.

BSIs are characterized by a low quantity of circulating microbes. On the basis of plating methods, the bacterial load has been estimated to be around 1 to 10 CFU per ml of blood in adults (105; 132; 133; 243; 251). This method is efficient to determine the amount of bacteria that survived the plating protocol; hence the true number of circulating bacteria is likely underestimated. DNA copies present in the circulation should be even higher as it also includes DNA released by dead bacteria or clumping bacteria as well as DNA from bacteria engulfed in phagocytes. On the basis of quantitative PCR, Bacconi *et al.* have recently estimated that during bacteremia, the blood contains 10^3 to 10^4 bacterial cells per ml (13), which is higher than the analytic sensitivity of most of the available molecular methods.

Hence, the ideal diagnostic method for BSI should be: 1) able to identify a broad range of pathogen, 2) have an analytic sensitivity lower than 10-100 CFU/ml, 3) rapid, 4) quantitative to give an idea of the severity of the infection, 5) give an antibiotic susceptibility profile, 6) associated with low hands-on time and 7) automated (65). These are all potential characteristics that molecular diagnosis methods may fulfil, at least to some extent.

Molecular methods for the diagnosis of BSI refer to nucleic acid-based methods but also to non-nucleic acid-based. PCR based methods that constitute the nucleic acid amplification-based methods can be applied both on positive blood culture and on whole blood. Indeed the PCR step increases the sensitivity

of the detection which makes it suitable for paucibacterial samples such as blood (Figure 1)(174). Nucleic-acid based methods non amplification based such as FISH (fluorescent n situ hybridization) or microarray have limited sensitivity. Hence, they are restricted to positive blood-culture. For the same reason, MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) a non-nucleic acid based method that analyse microbial protein mass-spectrum is suitable for diagnosis from positive blood-culture.

In this chapter we will present the molecular methods available for the microbial diagnosis of BSI. We will report and discuss the performance as well as the advantages and the inconvenient of these methods.

2. Blood culture-based diagnosis of bloodstream infections

Blood culture (BC) is currently the gold standard to establish the etiology of bloodstream infection. Blood bottles contain specific liquid broth for growth of bacteria or fungi present in blood. Distinct media are available to grow different microbes (174). BC-based diagnosis has been improved by the use automated incubators that can detect bacterial or fungal growth into blood bottles thanks to associated physicochemical variations. When the automated system detects the growth, visualisation of the microorganism is possible using standard Gram or fluorescent staining (figure 2). The Gram staining can give a first presumptive etiology of the BSI. However, only the final identification of the pathogen and its antibiotic susceptibility testing can insure the adequacy of the ongoing antibiotic treatment. Thus, when a blood culture is detected as positive, diagnostic laboratories have to identify the microbe rapidly and with the highest sensitivity and specificity. However, the time to positivity (TTP) of the blood-culture bottles can vary from few hours to 24-48 hours for fast growing bacteria, and even more for slow growing bacteria or fungi. In addition, it is generally admitted that more than 50% of BSI occur with negative blood culture (57; 79). When the blood culture become positive, using conventional methods, one or more days can be necessary to identify the pathogen and to determine the exact etiology of the BSI (figure 2). These methods include phenotypic characterisation using biochemical and enzymatic tests. Most of these

phenotypic characterizations require a subculture in liquid media or a subculture on solid media to obtain isolated colonies. Finally, BSI involving multiple organisms may also further increase the time to identification of most detection methods.

Inside positive BC, bacteria concentration reaches 1.10^6 to 2.10^8 for gram-negative cocci and 2.10^7 to 1.10^9 for gram-negative bacilli (42; 229). These concentrations allow the use of amplification-based methods such as PCR and real-time PCR as well as non amplification-based methods such hybridization or MALDI-TOF MS (figure 1).

Blood is a challenging sample for the detection and the identification of pathogens since it contains a low number of microorganisms in comparison to human components (DNA, proteins and cells) (175). These compounds can interfere with the detection or identification by leading to false positive or false negative (inhibition). All these components are transferred into the blood culture at time of the blood sampling. MALDI-TOF MS is a non-nucleic acid based molecular methods that has considerably accelerated and simplified the identification of pathogen from positive culture. MALDI-TOF MS is based on the analysis of the mass spectrum generated by bacterial proteins, mainly house-keeping proteins (figure 3). This technique is now applicable on positive blood culture but still requires a sample preparation step to discard blood and other non-bacterial components.

3. Microbial diagnosis of bloodstream infections directly from blood

The diagnostic of BSI directly from whole blood has been a major concern for medical diagnostic microbiologists. Indeed blood cultures appear to have some intrinsic limitations: 1) approximately fifty percent of BSI are BC negative (57; 79) and 2) in the case of positive BC, the TTP can vary from hours to days 3) BC requires a high quantity of blood that is difficult to obtain from some patients such as pediatric patients 4) an antibiotic treatment is often initiated prior to blood culture. Many technical improvements have been made to increase the performance of detection methods from whole blood but some limitations remained. Nucleic-acid based methods were limited by the need of large volume of

blood due to the low number of CFU per ml associated with the presence of human DNA in excess mainly due to white blood cells DNA (4). In addition, these methods are also sensitive to contaminant - bacterial or fungal DNA – or to the presence of DNA from dead organisms that could lead to false positive results (21; 162; 237; 238). Quantitative analyses are more powerful to interpret such positive results.

To improve the sensitivity of or nucleic acid based methods, the excess of human DNA should be removed. This could be achieved by removing white blood cells before nucleic acid extraction or by selective removal of human DNA after extraction. Nucleic acid based methods, in particular PCR, are sensitive to various inhibition mechanisms. Some of these mechanisms have been characterized but many of them remain unknown (112; 175; 195; 259). Inhibitory compounds can be contained in the sample or they can be the result of the sample preparation process. The blood contains some well known PCR inhibitors such as haemoglobin, bile salts and heme found in erythrocytes (2; 4; 111) as well as in lactoferrin found in leukocytes (4). The inhibition of PCR-based methods by these compounds is due to the release of iron (from heme) that is known to interfere with DNA synthesis (27). Immunoglobulin present in the bloodstream in particular IgG can inhibit PCR by binding single stranded DNA (3). Red blood cells present in blood bottles can also impair non-nucleic acid based methods such as MALDI-TOF MS as they bring a high amount of proteins that would interfere with the quality of the protein mass spectrum. Several protocols are now available for their removal prior MALDI-TOF MS analysis from positive blood bottles (51; 174; 253).

At present, each available molecular method is associated with a specific sample preparation. For the detection of a large number of organisms, the limited sensitivity of broad-range PCR targeting house-keeping genes have been overcame by the use of multiplex PCR or multiple real-time PCR. A recent technology, PCR/ESI-MS (PCR and Electrospray Ionization Mass Spectrometry) is a method that associates PCR and ESI-MS. The PCR allows the specific detection of low amount of bacterial, fungal or viral DNA from whole blood. This technology is associated with the analysis of PCR amplicon by ESI-

MS that determine the base composition rather than the nucleotide sequence. This has been shown to be sufficient for organism identification at the species level. Methods are now developed to remove the excess of non bacterial DNA and or inhibitors (4; 195; 202; 259). Most of these methods are time consuming, require intensive labour and are specific for one type of sample, one type of analysis or one type of device.

MOLECULAR METHODS FOR THE IDENTIFICATION OF PATHOGEN FROM POSITIVE BLOOD CULTURES

BC is currently the reference method for the identification of pathogens involved in BSI because it is easy to perform and sensitive due to the large volume of blood that could be analysed when multiple blood bottles are collected (174). When a BC is positive, conventional methods including phenotypic characterisation and rRNA gene sequencing have an extremely high specificity. However these methods are time consuming because they require an additional subculture and are challenged by a broad variety of molecular methods. Fluorescent in situ hybridization (FISH), the first molecular method that has been developed, is based on the binding of specific probes on pathogens DNA. FISH is a rapid, sensitive and specific method but is dependent on the choice of the probes to be tested. Microarrays allowed the detection and identification of pathogens as well as resistance genes. PCR-based methods allow the rapid detection of a single organism (specific real-time PCR) or multiple organisms (broad-range PCR or multiplex PCR). These methods are highly sensitive to contaminating DNA or PCR inhibitors. MALDI-TOF MS, one of the latest commercialized technologies is presented as a revolution for clinical microbiology laboratories (18). Indeed, MALDI-TOF MS may identify in less than one our most bacterial and fungal agents generally recovered from BCs.

1. Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization (FISH) is based on the specific binding of fluorescent peptide nucleic acid probes (PNA) to the rRNA – 16S rRNA for bacteria and 18S rRNA for fungi and other eukaryotes. Basically, slides are prepared from a positive blood culture, the fluorescent PNA are applied and unbound PNA are removed by a washing step. Bound fluorescent PNA is generally observed using a fluorescent microscope as flow cytometry detection still need further investigation and development (11). FISH requires a high number of living cells which prevent its use directly on whole blood. Multiples probes that recognize microorganisms at the genus level or at the species level are available. All the solutions are based on multiple probes that detect and distinguish two or three organisms. A first kit of the PNA-FISH system (AdvanDx, Wolburn, MA) allowed the detection of *S. aureus* and coagulase negative staphylococci (36; 86; 93; 104; 173). A second kit allows the detection of *Enterococcus faecalis* versus *Enterococcus faecium* and other *Enterococcus* spp. (OE) (87). *E. coli* and *P. aeruginosa* can be detected using a PNA mix or using a mix of three probes that include also *K. pneumoniae* (183; 189; 220). *C. albicans*, *C. glabrata*, *C. parapsilosis and C. krusei* can also be detected (74; 100; 199; 215; 255).

The Quick-FISH system is a more rapid version of the PNA-FISH system. The turnaround time (TAT) is shortened by reducing the hybridization phases to 15 min and by removing the wash step. At present, the QuickFISH system provides kits that detect and distinguish *S. aureus/*Coagulase negative staphylococci, *Enterococcus faecalis/Enterococcus faecium*, *E. coli/P. aeruginosa* (55; 56; 158). The diversity of microorganisms that is currently detected is still limited but these bacterial species represent about 95% of the pathogen involved in BSI. The choice of the probe to be tested will depend on the Gram result, the clinical presentation and the local epidemiology. The sensitivity of the QuickFISH system is 99.5% for the detection of *S. aureus* and 98.8% for coagulase negative staphylococci, with a combined specificity of 89.5% (55). Martinez et al. reported 97.9% of concordance with conventional detection methods for Gram-positive bacteria and 95.7% for gram-negative bacteria (158). Yeast (*C. albicans*, *C. parapsilosis*,

C. tropicalis, C. glabrata and C. krusei) can also be identified using probes targeting 26S rRNA sequences (74). Commercial methods give a result in 1.5 to 3 hours with high sensitivity and specificity, but could not replace other detection methods as many significant pathogens are not detected using these probes (table 2) (31; 87; 158; 242).

2. Microarray

Microarrays are based on species-specific or genus-specific DNA probes immobilized on chip on which microorganisms DNA will specifically hybridize. The high-density of the chips allow the use of sequences specific for different pathogens and probes for the detection of virulence factors and/or resistance genes. Species identification is determined by the pattern of hybridization and on the intensity of the signal (47; 179; 254; 264). The analytic sensitivity of microarrays ranges between 10 to 10^5 CFU/ml depending on the pathogen, which allows their use from positive BC but not directly from blood (254). The Verigene Gram-Positive Blood Culture system and the Verigene gram-negative Blood Culture system (Northbrook, IL, USA) can detect gram-negative or gram-negative microorganisms respectively, as well as associated resistance genes. The time to identification is approximately 2.5 h from positive blood culture bottles (table 2). After being extracted from 350 µl of blood culture from positive vial, the bacterial DNA is suspended in a specific buffer and hybridized on specific synthetic oligonucleotides followed by a second hybridization step involve gold particles. The signal is further amplified via a silver staining process which increases the sensitivity of the system. The reading is automated. The Verigene Gram-Positive Blood Culture system can detect 9 organisms at the species level (*Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Streptococcus anginosus* group, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis* and *Enterococcus faecium*), 4 genus (*Staphylococcus* spp, *Streptococcus* spp., *Micrococcus* spp. and *Listeria* spp.) and detect 3 resistance genes (*mecA* (methicillin), *vanA* (vancomycin) and *vanB* (vancomycin). Studies performed on both adult and pediatric patients reported 89.7% to 99% of concordance for the detection

from positive BC between the Verigene Gram-Positive Blood Culture system and the traditional method (5; 14; 158; 165; 206; 227; 258). Misidentifications have been reported for *Streptococcus* spp.. In particular group mitis *Streptococcus* spp. have been misidentified with *S. pneumoniae* and *S. oralis* have been misidentified with *S. anginosus* (206). Performances of the Verigene system are higher on mono-bacterial samples. Mixed culture can prevent the identification of one of the pathogen. Buchan et al reported 98.6% of sensitivity and 94.3% of specificity for the detection of the *mecA* gene in 5999 *Staphylococci* cultures, and 100% of sensitivity and specificity for the detection the *vanA* gene in 81 cultures containing *Enterococcus faecalis* and *Enterococcus faecium* (26). Samuel et al reported 91% of concordance for *mecA* and 100% for *vanA* detection when compared to routine methods (206). In case of mixed population of staphylococci, the resistance gene cannot be associated to a specific pathogen (26). The Verigene Gram-Negative can detect 5 pathogens at the species level (*Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Serratia marcescens*) and 4 genus (*Acinetobacter* spp., *Citrobacter* spp., *Enterobacter* spp. and *Proteus* spp.). The Verigene Gram Negative BC assay displays 91 to 100% of agreement for the detection of gram negative pathogens both from adult and pediatric patients (17; 60; 108; 155; 226). Among, the false-negative results, mixed blood cultures have been incriminated; but some of these negative or non called results involved bacteria covered by the chip (60). As for many molecular assays, it is not possible to distinguish *E. coli* and *Shigella* spp. (*S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*). This distinction should be achieved with enzymatic analysis. The Verigene Gram Negative assay can detect 6 resistance genes: the extended-spectrum beta-lactamase (ESBL) CTX-M and the carbapenemases KPC, NDM, VIM, IMP and OXA groups with 92.3% to 100% of concordance with routine methods (60; 234). Other microarrays for the detection of resistance gene are currently being developed (24).

The performances of microarrays are extremely high for both the detection of pathogen and resistance cassettes when applied to positive blood cultures but supported by a limited number of studies.

3. Nucleic acid amplification-based methods

PCR provide a rapid and specific technology for pathogen identification from positive BC (260). The specificity provided initially by the use of specific primers can be increased in the case of real time PCR, by the use of specific probes. The use of PCR on blood or on hemorrhagic samples, was limited for long-time by the fact that PCR is sensitive to the presence of inhibitory compounds such as haemoglobin contained in erythrocytes (2; 4; 111), lactoferrin contained in leukocytes (4), or immunoglobulin (3). To avoid inhibition, recent methods use new or improved nucleic acid extraction and/or amplification techniques.

Multiplex-PCR increases the time to result as it interrogates several targets (pathogens or resistance genes) at the same time. In addition it can increase both the specificity and the sensitivity of an analysis thanks to the use of multiple targets for the same organism (120; 223). Multiplexing is achieved through the use of several specific primers pairs in the same reaction. Different methods can be applied to identify the amplified sequence(s): 1) the use of specific probes labelled with distinct fluorochromes, 2) the analysis of the probe or the amplicon melting curves (69; 72; 257); 3) the amplicon size (electrophoreses); 4) the sequencing of the amplification product. The latest technology, PCR/ESI-MS, is based on the analysis of the amplicon by MS (Figure 5). The latter technique will be discussed in depth in the paragraph on pathogen detection from blood.

Multiplex PCR

The FilmArray system (Idaho Technology, Salt Lake City, UT, USA) using multiple-PCR is a solution that allows the identification of more than 25 pathogens and 4 antibiotic resistance genes from positive BC in 1 h (19). The FilmArray is a closed system that uses multiple-PCR expected to identify 90 to 95% of the pathogens involved in BSI as well as the resistance genes *mecA*, *vanA* and *vanB* and bla_{KPC}. A kit

for the identification of potential bioterrorism agent (*Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis*) is also available (249).

In a prospective study on 102 blood cultures, the FilmArray system displayed 91% of sensitivity and 77% of specificity when compared to conventional identification methods for the detection of pathogen present in the FilmArray panel only (19). The detection of resistance genes revealed 100% of specificity and 100% of sensitivity in this study. In another study, FilmArray displayed 98.5% of sensitivity and 100% of specificity for the identification of gram-negative bacteria, 96.7% of sensitivity and 93.7% of specificity form gram-negative bacteria and 96% of sensitivity and 98.9% of specificity for the detection of the *mecA* gene. Another study performed on 118 mono-bacterial culture reported 92% of correct identification of the FilmArray system, but mixed cultures gave no results (17). The identification of *Candida albicans* and *Candida glabrata* reached 100% of sensitivity and 99.5% of specificity (6). This is consistent with other study showing high performance of the FilmArray for the identification *C. albicans* and *C. glabrata* (181). The FilmArray can also be applied on other sterile samples such as CSF (184).

Rapid PCR-based systems

The clinical and epidemiological impact of pathogens such as methicillin resistant *Staphylococcus aureus* (MRSA) or rifampicin resistance *Mycobacterium tuberculosis* has contributed to the development of methods for the rapid detection of resistance. *S. aureus* is a significant agent of community-acquired and nosocomial infections (256). The mortality rate is increased in patient infected with MRSA in comparison to patient infected with MSSA (methicillin sensitive *S. aureus*) (49; 50; 263). The increased percentage of MRSA in comparison to MSSA has stressed the importance of rapid detections methods of MRSA (71; 77; 169; 170). Several methods based on multiplex real-time PCR are available: the GeneXpert MRSA/SA BC Assay (Cepheid, Sunnyvale, CA) (45; 221), the StaphSR assay (BD GeneOhm, San Diego, CA) (117; 222) and the StaphPlex (Genaco Biomedical Products, Huntsville, AL, USA) (230). The GeneXpert and StaphSR assay are rapid PCR-based systems developed for the detection of *S. aureus*

only in approximately 1.5 h. They require only limited hands-on time and skills. The application of the GeneXpert MRSA on positive blood culture was shown to have an impact on anti-infectious treatments by reducing the use of glycopeptides (46). Alternatively, the StaphPlex system can identify several staphylococci at the species level. The StaphPlex system is based on several PCRs (18 target genes) and microarray analysis for the identification of staphylococci at the species-level, the detection of resistance genes and of the Panton-Valentine leukocidin (PVL). The results are obtained within 5 hours (230). Because these methods are largely aimed to detect resistance genes, they will be presented and discussed in the chapter dedicated to resistance detection.

4. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

Principle of MALDI-TOF MS

The MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry) is a method that allows identification of a broad range of microbes at the species or at the genus level from positive culture. The method was first developed to be used on colonies before its application on BC. Microbe identification using MALDI-TOF MS is based on the analysis of the mass spectrum generated by bacterial or fungal component, mainly house-keeping proteins (the ribosomal proteins which are basic are specifically extracted using acidic matrix). This spectrum is a unique fingerprint for each microorganism but displays features shared between genetically related bacteria (44). The comparison of the spectrum with a database of spectrum obtained for characterized organisms allow microbes identification at the species or at the genus level depending of a score assigned by the identification software (52).

MALDI-TOF MS was first used for bacterial identification in 1975 (8). The routine use of mass spectrometry in diagnostic laboratory for the identification of bacteria was proposed in 1996 when correct

identification has been achieved directly from whole bacteria coming from single colonies (44; 110). Identification of bacteria using the MALDI-TOF MS is divided in three steps: (1) ionization of the sample, (2) separation of proteins in a flight tube (3) generation of a mass spectrum by determination of the mass of the proteins (figure 3). The sample preparation consists in mixing the cells from a bacterial colony, with a crystallizing matrix that will trigger the ionization of the sample achieved by a laser (MALDI). Protein accessibility can be facilitated by a quick formic acid extraction. Ionized proteins are accelerated by an electrostatic field and separated along a flight tube. The separation is due to the time of flight (TOF), a function of the mass and of the charge of the proteins. Protein detections will generate a mass spectrum unique for a defined bacterial strain corresponding to mass-to-charge ratio (*m/z*) between 1000 and 20000 kDa. However, several peaks are shared between bacteria from the same genus or species and serve as biomarkers for bacterial identification. The identification is performed by comparison of the mass spectrum with a database of spectra obtained from characterized bacterial strains. The software that performed the spectral analysis and the comparison with the database, assign a score for the identification. Depending on the threshold recommended by the furnisher and the in-house algorithm, the identification can be rejected, or accepted either at the species level or only at the genus level.

Application of MALDI-TOF MS on positive blood culture bottle

In clinical microbiology, the MALDI-TOF MS was first used on pure bacterial colonies before its application on positive BC for the diagnostic of BSI (figure 3) (52). The initial analysis on a positive blood culture bottle is a Gram staining. The Gram staining is especially recommended 1) to confirm that the detection is truly due to the presence of a microorganism and 2) give a presumptive etiology of the BSI and 3) to disclose mixed bacteremia. In positive blood cultures, the bacterial concentration is ranging from 10^6 to 10^9 CFU/ml, which in theory is sufficient for MALDI-TOF MS identification. However, the presence of high amount of non bacterial material (erythrocytes, nutrient from the growth media) impairs the direct identification from the blood vial.

Application of MALDI-TOF MS after a short subculture

A short subculture (2 to 3 hours) on agar plates can be performed from the positive blood vial to obtain a thin layer of bacteria (174). From this thin layer, the MALDI-TOF MS analysis can be performed (figure 2) (116; 239). Short subcultures are also adequate for AST using automated systems (116). However, this procedure is mainly suitable for fast growing bacteria.

Application of MALDI-TOF MS directly from the blood culture vial via a bacterial pellet

The fasted method is to perform the identification directly from the positive BC (figure 2 and figure 3). The goal is to get rid of non bacterial components present in the bottles such as red blood cells and to concentrate the bacteria. Bacterial concentration can be achieved by centrifugation and erythrocyte lysis by ammonium chloride (51; 193). This generates a pellet that can be identified by MALDI-TOF MS (figure 3). This method allowed the identification of 78.7% of the samples obtained. Moreover, 99% of the MALDI-TOF MS identifications were correct at the species level (193). Alternative erythrocytes lysis techniques can be achieved with formic acid (136). Any methods implying mild detergent that would solubilise erythrocytes membrane but not microbe membrane lead to similar results (83; 163; 168). Alternatively, gel-based separator tubes have proven efficiency in concentrating bacteria and removing red blood cells (224).

MALDI-TOF MS has a major impact on the time to result since it can be achieved directly from positive blood culture, without subculture (61). The sample processing and identification using MALDI-TOF MS takes approximately 1 hour, which makes it time effective for fast growing bacteria as well (136; 193).

The accuracy of the MALDI-TOF MS identification is dependent on extended and correct spectrum database. This database can be implemented with spectrum obtained from clinical isolates. This is applicable for strains for which the MS identification failed and that were identified without any doubt with other methods such as gene sequencing or enzymatic assay. This is also applicable for rare

pathogens that can be identified faster and accurately using MALDI-TOF MS (176; 213). However, some closely related bacterial species such as the different *Streptococcus* species remain difficult to distinguish using MALDI-TOF MS. For instance, group mitis *Streptococcus* are often misidentified as *S. pneumoniae*. *S. pneumoniae* identification should thus be confirmed by phenotypic test such as optochine susceptibility (126; 224; 227). Similarly, the distinction of *E. coli* and *Shigella* sp. is impossible using routine MALDI-TOF MS procedures and requires additional phenotypic confirmation (59; 210). Recently, a new approach based on the analysis of biomarker peaks has been proposed to differentiate these two closely related pathogens (128).

Efficiency and reproducibility of MALDI-TOF MS identification

Even if a mass spectrum can be obtained from a single colony, this biomass represents a significant amount of bacteria. MALDI-TOF MS identification is therefore not applicable directly from blood but is dependent on microorganisms proliferation in BC and on an additional concentration step from the positive BC (174). The requirement of a culture limits the use of the MALDI-TOF for the detection of non-cultivable bacteria.

Proteins used as biomarkers for MALDI-TOF MS identification of bacteria are mainly involved in house-keeping functions (246). Ribosomal proteins contribute to approximately half of the peaks present in the mass spectrum (10; 201). This makes it reproducible and robust as several peaks would be conserved in genetically related bacteria. Nevertheless, experimental conditions can impact the presence and the relative abundance of detected peaks. This is true for both routine identification or for the implementation of the peaks pattern into the in-house database. The culture media, the growth conditions and the age of the colony may impact the spectrum (235). The presence of agar residue or blood proteins in the sample may impact the spectrum quality (52). Quality of the matrix may also influence the spectrum (246).

Importance of the Gram staining and of the subculture

The Gram staining on positive blood culture is still mandatory (i) to confirm the presence of a microbe in the bottles (ii) to validate the identification of the MALDI-TOF MS, which should be congruent with the characteristic provided by the Gram (88) (iii) to disclose polymicrobial infections as the MALDI-TOF MS poorly identify mixed bacterial samples. Mixed infection may also be detected upon subculture, which also remains essential for the antimicrobial susceptibility testing (see later).

MOLECULAR METHODS FOR THE DETECTION OF PATHOGENS DIRECTLY FROM BLOOD

BSI is initially suspected by clinicians on the basis of clinical signs and symptoms. At this stage the etiological agent is however difficult to suspect as the clinical presentation is generally similar. To accelerate the time to result of microbial diagnosis, molecular methods that can be used directly on whole blood have been proposed. Nevertheless, blood as sample presented many technical limitations: 1) the low quantity of circulating microbes during BSI (1 to 10 CFU per ml), 2) the presence of PCR inhibitor in blood that are not completely removed by current nucleic acid extraction methods *(see Introduction section*), 3) the sensitivity of PCR to contamination; which implies the use of highly pure (nucleic acid free) material and ideally to perform quantitative analyses (211), 4) the ability of PCRs to detect the presence of DNA from both living and dead microbes which makes the interpretation of positive results difficult (BSI *versus* DNAemia and 5) the presence of human DNA in excess .

Different PCR technologies are now available. Broad-range PCRs could be useful, especially when the etiological agent cannot be suspected, but they are often limited by lower sensitivity. Pathogen specific PCRs display higher sensitivity. Real-time PCRs can be multiplexed to detect several targets at the same

time with a good sensitivity. A recent innovation that can also be use directly on whole blood is PCR-ESI-MS that is presented as a universal and fast method.

1. Broad range PCR

The need for a PCR-based universal detection method is stressed by the importance of rapid diagnostic in the setting of critically-ill patients, especially since the etiology of the BSI is difficult to establish on the basis of the clinical presentations. This is the case for neonate patients with the additional difficulty that only small volumes of blood are available, which decreases significantly the sensitivity of BC. Alternatively, such universal and sensitive methods may be used to screen blood samples taken from neutropenic patients or other high risk subjects to early detect the etiological agent of a BSI.

The 16S rRNA gene that displays sufficient level of conservation among bacterial species is the target of in-house PCRs used in many molecular diagnostic laboratories (12; 40; 41; 43; 94). In many organisms, the ribosomal operon is present in multiple copies which increases the sensitivity of PCRs targeting this genomic region. The intergenic spacer is more polymorphic and more species specific. Specific PCR primers are designed on conserved regions that surround highly variable region. Thus, the sequencing of the amplicon allows the identification of the microbe at the genius or at species level. Most of these PCR cannot be used directly on blood because of their limited sensitivity and specificity. However, some studies have reported sensitivity of PCR targeting the 16S rRNA gene ranging from 10 to $2.5.10^2$ CFU per reaction directly from blood (131; 191). Gaibani *et al* have developed a broad-range real-time PCR that targets a 97 base pair sequence of the 23S rRNA gene. This PCR is expected to detect 90% of the bacteria involved in BSI but does not give any identification, since the short fragments amplified do not allow discrimination upon sequencing (89). This real-time PCR can be used on whole blood (100 µl). Its sensitivity is ranging from 10 to 10^3 CFU per reactions for *E. coli* and *S. aureus*, depending on the extraction method.

The SepsiTest system (Molzym, Bremen, Germany) is based on broad-range PCR using universal primers that target the 16S rRNA gene for bacterial identification and the 18S rRNA for fungal identification. The amplicon are sequenced and analysed by BLAST to give identification at the species level (=> 99% of identity) or genus level (=> 97%) (134; 250). Of course this cut-off does not apply to all bacterial species and should be used with caution. The analysis is achieved from 1ml of whole blood. After lysis of human cells, the human DNA is degraded by a DNAse. The PCR is achieved using reagent provided by the manufacturer and lead to amplicon of about 450 base pairs. The time to result is approximately 6 hours. Depending on the study, the sensitivity and specificity are variable when compared to BC. A first study performed in critically-ill patients led to 28.6% of sensitivity and 85.3% of specificity of the SepsiTest when BC is used as gold standard (145; 212). In a second study, pathogen could be detected in 26 % (13/50) of the critically-ill patients while SepsiTest could detect pathogens in only 12 % of the patients (212). In a third study, still using BC as gold standard, SepsiTest sensitivity was 21% and specificity was 96% (152). In contrast, high sensitivity and acceptable specificity (87% and 85.5%) was monitored in a multicenter study involving 342 blood samples from 187 patients (250). In a study performed on patients supported by extracorporeal membrane oxygenation, the sensitivity and specificity were 78.6 % and 88.4 % when compared to BC with 97.7% of concordance of positive results (177; 200). In this study, SepsiTest could detect at least a pathogen in 25% of patients with negative BC. For patient with suspected endocarditis, Kuhn *et al.* reported higher performances of SepsiTest over BC (134). The variable sensitivity and specificity of the SepsiTest when compared to BC needs to be investigated. One specific feature of SepsiTest is that it can be used on sterile samples other than blood with the potential to replace home-made broad-range PCR (96; 200). A limitation of this method is that the extraction is not automated (99). Another important limitation of all these molecular tests is that they do not provide strain for detailed antibiotic susceptibility testing.

The VYOO system (SIRS-Lab, Jena, Germany) is based on multiplex PCRs that can detect 34 bacteria and 7 fungi from 5 ml of whole blood. The system contains several steps, including non-automated steps.

Nucleic acid extraction is assisted by magnetic beads. Then there is a step of enrichment in microbial DNA based on i) the methylation difference between microbes (bacteria and fungi) DNA and the human DNA and ii) the use of chromatography affinity (202). The PCR is achieved on DNA which concentration has to be manually adjusted to 1 µg in 25ul. Two PCRs with a specific pool of primers are required. The amplicons are applied to an electrophoresis in an ethidium bromide stained agarose gel. Pathogen identification is determined on the basis of the band pattern.

In a study performed on 311 blood samples from 245 patients of an ICU of a university hospital analysed in parallel with BC and with the VYOO system, the VYOO system gave 30.1% of positive samples, with a median time to result of 24.2 hours and BC bottle gave 14.5% of positive samples with a median time to positivity of 68 hours with only 40% of correlation between the two methods (22). PCR positive samples correlated well with the level of procalcitonin. Additionally the PCR results from blood were most of the time confirmed by the pathogen identified in the suspected site of infection. Importantly 34% of the patients with positive VYOO test, the anti-infectious therapy was inadequate, including infections that involved VRE, multi-drug resistant staphylococci and fungi (22). In another study, the VYOO gave positive results in 51.4% of samples from patient with BSI, when BC gave 27.7% of positive results (84).

These studies suggest that VYOO is more sensitive than BC, which could be explained by the association of a large starting volume (5 ml) and the enrichment in microbial DNA before amplification. More studies are required but these preliminary performances are promising despite the fact that many steps are not yet automated and that.

2. Real-time PCR and Multiplex PCR

The multiplexing of real-time PCRs allows detecting simultaneously several microbes with a good sensitivity and specificity. This is the technology used for the LightCycler SeptiFast system (Roche Molecular System, Germany), the MagicPlex Sepsis system (Seegene, Seoul, Korea).

Principle of the LightCycler SeptiFast system

The LightCycler SeptiFast system is based on broad-range real-time PCR that can identify 19 pathogens (8 gram-negative bacteria, 6 gram-negative bacteria and 5 fungi) representing approximately 90% of the pathogen responsible for BSI. The SeptiFast can be used directly from whole blood with an overall time to result of about 5 hours (3.5 when associated to an automated extraction system). The targets of the multiplexed broad-range real-time PCR are the internal transcribed spacer (ITS) between the 16S and the 23S ribosomal DNA for bacteria and the ITS between the 18S and the 5.8S ribosomal DNA for fungi. Identification at the species level is determined by the distinct melting curves of the specific probes. The LightCycler can detect 8 gram-negative bacteria detected: *E. coli*, *Klebsiella pneumonia*/ *Klebsiella oxytoca*, *Enterobacter cloacae*/ *Enterobacter aerogenes* species, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*. Six Gram-positive bacteria can be recognized: *S. aureus*, coagulase negative staphylococci, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus agalactiae* / *Streptococcus mitis*, *Enterococcus faecium* and *Enterococcus faecalis*. Six fungi can be detected and identified at the species level: *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida krusei* and *Aspergillus fumigatus*. Basically, the assay consists of three separated reactions that contain distinct primers and probes mixes to detect respectively gram-negative bacteria, gram-negative bacteria and fungi. The LightCycler SeptiFast is designed for the analysis of 1.5 ml of blood on which a mechanic cell lysis is achieved with the SeptiFast Lys KIT MGRADE and the MagNALyser from Roche diagnostic prior DNA extraction using also a commercial kit provided by the same manufacturer. The assay contains an internal control that consist of a synthetic double-strand DNA, similar to the expected amplicon but with a distinct probe binding site.

The analytic sensitivity is ranging from 3 to 30 CFU/ml for bacteria and is 100 CFU/ml for fungi (34; 252). The experimental sensitivity (42.9% to 95%) and specificity (60% to 100%) of the SeptiFast are variable depending on the studies and on the patients characteristics (table 1) (21; 35).

Diagnosis of Infection in febrile neutropenia using the LightCycler SeptiFast

From a group of 86 febrile neutropenic patients representing 141 episodes of fever, BC and SeptiFast detected approximately the same number of microorganisms: 44/141 (31.2 %) and 42/141 (29.8%), respectively. However, the association of BC and SeptiFast increases the rate of documentation from about 30% to 43% (61/141) which might be due to the fact that only 12 organisms where detected by both BC and SeptiFast (139). A similar observation was made by Mancini *et al.* on a study performed on 103 samples from neutropenic patient with haematological diseases in which 20.4% of the samples were positive with BC and 33% using SeptiFast, with only 83% of correlation between the two methods (154). For Bravo *et al.*, the agreement between BC and SeptiFast was 69% for neutropenic patients and 75% for patients from the intensive care unit (25). All these studies suggest that SeptiFast cannot replace BC but that the two techniques could be complementary (98; 139; 154; 161). Lamoth and colleagues suggested that the low sensitivity of SeptiFast was due i) to organisms absent from the SeptiFast analytic spectrum (40% of false negative) and ii) to the cut-off that decrease the sensitivity for the detection of coagulase negative staphylococci or for streptococci and increased the rate of false negative (139). False negative results obtained for gram-negative cocci might also be explained by the inefficient lysis of these microorganisms (182) or to PCR inhibition in the case of high bacterial load. The adjustment of the experimental procedure and an adequate cut-off might increase the performance of the SeptiFast assay for neutropenic patients (139; 182). In a context of persistent fever, SeptiFast identified new pathogens in 89% of the cases whereas BC identifies 8% of the pathogens (139). Similar results were obtains by von Lilienfeld-Toal *et al.* (241). In this study all the patients with a probable fungal infection had positive SeptiFast results for *Aspergillus fumigatus*. This suggests a potential added value of the SeptiFast assay for the detection of fungemia in neutropenic patients (35; 139; 154; 180; 241).

Diagnosis of infectious endocarditis

A study performed on 63 patients with suspected endocarditis revealed a low sensitivity of the SeptiFast when compared to BC (34). Among 19 patients with positive blood culture at their admission, SeptiFast detected 8 bacteria (41% of sensitivity). Twenty-two patients had positive blood culture before their admission but because of efficient antibiotic treatment they had negative blood culture at the time of this study. Among them, SeptiFast detected only 3 bacteria (3/22) (34). In this study, SeptiFast did not detect any microbes for patients without any etiology (100% of specificity) (34). Because the low sensitivity of the SeptiFast was not due to pathogens that are not detected by this system, it could be due as discussed later to a low bacterial load. In another study involving 20 patients with endocarditis, SeptiFast performed on excised cardiac valves, displayed a higher sensitivity (95%), and specificity (100%) than culture (sensitivity 15% and specificity 100%) (81; 147). In patient with suspicion of *Candida* spp. endocarditis, SeptiFast was as sensitive as BC to detect fungal infection (141).

Diagnosis of neonatal sepsis

In a study performed on 1,673 pediatric samples (803 children), the detection rate of infection was higher with the SeptiFast (14.6%) than with BC (10.3%) which corresponded to a sensitivity of 85.0% and a specificity of 93.5% of the SeptiFast (153). The cumulative positive rate of BC and SeptiFast was 16.5%. Another study confirmed a higher sensitivity but a lower specificity of SeptiFast for the detection of late-onset neonatal sepsis (127).

Advantages and inconvenient of the LightCycler SeptiFast system

The performances of the SeptiFast are variable depending on the studies. The overall sensitivity of the SeptiFast is not higher than the sensitivity of BC but together SeptiFast and blood culture increase the rate of microbial documentation of BSI. This suggests that the SeptiFast cannot replace blood cultures but that these two methods are complementary. One of the major advantage of the SeptiFast is the time to result

(<5hours). In a study performed on 114 consecutive patients with clinical evidence of sepsis the mean time to results for SeptiFast was less than 8 hours when BC mean time to positive result was 3.5 days and for negative result 5 days (218). In a study performed on patients of the emergency department with suspected sepsis, Schaub *et al.* Reported a median time to positivity of BC of 16 hours (without the organism identification) (209). SeptiFast might be useful in case of a persistent bacteremia, for instance in neutropenic patient with persistent fever, for which SeptiFast often identified additional pathogens. There are a high number of studies on the performance of the SeptiFast. However, there is a need of interventional studies to clearly determine the clinical impact of this new technology.

One of the limits of the SeptiFast is also the absence of exact quantification. Cut-off can be used for the interpretation of streptococci and coagulase negative staphylococci positive results in particular. This is expected to reduce the number of false positive results but might prevent the detection of low grade infections. In some studies performed in neutropenic patients, the sensitivity of SeptiFast for the detection of coagulase negative streptococci is decreased, which could be due to the fact that the cut-off were established for non-neutropenic patients. In contrast in pediatric studies SeptiFast leads to increasing number of false positive due to coagulase streptococci. Adding quantitative data to the SeptiFast has the potential to predict the severity of the sepsis (266).

The HACEK group of fastidious bacteria (*Haemophilus, Actinobacillus, Cardiobacterium, Eikenella* and *Kingella*) involved in a significant number of blood culture-negative bacteremia is not covered by the SeptiFast. For this reason SeptiFast is not sufficient by itself to diagnose all the bacteremia and alternative methods will still be required to cover the entire bacterial kingdom. Finally, in the context of an increasing number of multi-drug resistance organisms, it is a limitation that SeptiFast does not provide any data on the resistance profile of identified organisms excepted for MRSA but using an additional kit (table 1).

The MagicPlex Sepsis system

The MagicPlex Sepsis system (Seegene, Seoul, Korea) is based on multiplexed real-time PCRs that can detect 90 pathogens at the genus level, 25 at the species level (19 bacteria and 6 fungi), and the resistance genes *mecA*, *vanA* and *vanB* directly from whole blood (1 ml). A specific nucleic acid extraction kit is used to enrich in microbial DNA. A first amplification is a screening that provides either (i) an amplicon bank that consists of gram-negative bacteria and fungi or (ii) an amplicon bank that consists of gram-negative bacteria (n=73) and resistance genes conferring resistance to methicillin (*mecA*) or vancomycin (*vanA* and *vanB*) . A second step is a screening for bacteria and fungi at the genus level and for the presence of resistance genes (time to result 5 hours).

The performance of the MagicPlex system was compared to BC on 267 patients from the intensive care-unit, from the haematology department and from the emergency department of a tertiary hospital, which revealed an agreement between the two methods of 73 % with no statistical difference between their sensitivity (30). For patient with antibiotic treatment, the sensitivity of the MagicPlex 65% was lower than the sensitivity of BC (71%); specificity was respectively 92% and 88% for each method. Another study performed on 140 patients with suspected BSI reported 37% of sensitivity and 77% of specificity of the MagicPlex when considering BC as gold standard (152).

Additional studies are required to determine the exact potential of this device. This method is limited by the number of pathogen detected at the species level and by the absence of quantification. Another limitation is the absence of automation and the need of a specific extraction method which might makes it difficult to integrate in an automated molecular diagnostic laboratory.

3. PCR-Electrospray Ionization Mass Spectrometry (PCR/ESI-MS)

Principle, characteristics and performance of PCR/ESI-MS

PCR-Electrospray Ionization Mass Spectrometry (PCR/ESI-MS) is a technology that was initially developed to face bioterrorism threats or to run public health investigations. The aim was to provide a rapid method for the detection and identification of pathogens from various type of sample. The technology had to detect low quantity of a given pathogen even in polymicrobial samples and should include the detection of non cultivable or fastidious microorganisms. In addition, the technology should be able to detect known microorganisms as well as yet unknown pathogen. Molecular diagnosis was chosen as it is fast and broad range.

The PCR/ESI-MS approach is based (i) on the amplification of microorganisms DNA by multiple PCRs and (ii) on the identification of the organism(s) at the species or genus level through the analysis of the amplicon by ESI-MS (Electrospray Ionization Mass Spectrometry) (65; 123). Basically, PCR/ESI-MS consists in 5 steps: 1) extraction of the microorganism or sample DNA, 2) amplification of the DNA using multiple pairs of primers, 3) precise determination of the molecular mass of the amplicon(s) using ESI-MS, 4) deduction of the base composition of the amplicon(s) from the exact MW of the amplicon 5) identification of the pathogen(s) by integrating the informations obtained from several amplicon(s) (figure 4) (66; 123).

Before being applied to blood (see next paragraph) PCR/ESI-MS was first applied on bacterial colonies and on environmental samples. Practically, DNA amplification is based on multiple broad-range PCRs, which is more sensitive, than a single broad-range PCR based on degenerated primers. The primers are targeting conserved genomic region surrounding polymorphic regions (for instance DNA regions encoding for the 16S or the 23S rRNA). Moreover, these PCRs are devoid of fluorescent dye or probes, which allows high-level of multiplexing. DNA amplification may be performed in 96 well plate using the

following steps: 1) 95°C for 10 min, 2) 8 cycles of 95°C for 30 s, 48°C increasing of 0.9°C at each cycles for 30 s and 72°C for 30 s 3) 37 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 20 s 4) final extension of 2 min at 72°C and hold at 4°C hold (73). Major innovation of PCR/ESI-MS is that the pathogen's identification relies only on the base composition (A, C, G and T) of the amplicons (65; 123). The base composition is obtained by the integration of the exact mass of the amplicon, the length of the amplicon, the mass of each base and the complementarily rules of DNA (figure 4). The choice of the PCR targets makes the base composition of one or more amplicon(s) sufficient for pathogen identification. The absence of Sanger sequencing dramatically reduces the time to result(s). Thus, ESI-MS provides the base composition of an amplicon in about 1 minute, the mass of the amplicon being determined by the time of flight (TOF). The base composition of the amplicon(s) is compared to a database providing the identification at the genus or at the species level with a score of probability (Q-score) inferred by an ESI-MS triangulation software relying on multiple amplified regions. The Q-score integrates multiples parameters such as the number of primer pairs that gave an amplicon, the number of potential microorganism, the proximity of the base compositions to reference matches in the database.

A set of 9 pairs of primers is used for the coverage of the bacteria kingdom. Four pairs are necessary for *Candida* species. Four additional pairs of primers have been designed to detect the resistance cassettes *mecA*, bla_{KPC}, *vanA* and *vanB*. Using clinical samples (up to 1.25 ml) other than blood, PCR/ESI-MS displays good sensitivity and specificity. This includes environmental samples as well as clinical samples such as CSF (75; 76; 171) and respiratory tract samples (64; 91; 119). Compared to culture the PCR/ESI-MS correctly identified 95.6% and 81.3% of the strains at genus level and species levels respectively. Among 395 respiratory samples, PCR/ESI-MS displayed 67.6% of agreement at the genus level and 66.6% at the species level with culture. PCR/ESI-MS was able to identify fungi in 20.3% (35/172) of the respiratory specimens with a negative culture suggesting a better sensitivity than culture (217). A commercial kit available for the detection of viruses (38; 39; 48; 107; 135; 142; 185; 228; 231) has been shown to display higher performance than real-time PCR and microarrays (228). PCR/ESI-MS have also

been successful at identifying *Mycobacterium* species at the species level and at determining associated resistance genes using a panel of 8 PCRs (138; 160; 245).

Thus, PCR/ESI-MS represents a universal method that may be applied to bacteria, viruses and fungi and that is expected to also identify any unknown species. Indeed, when primers are designed to identify all known members of characterized groups, they allow the detection of unknown or new emerging pathogens from these groups (138; 203; 204). This provides a potential for the rapid detection of emerging pathogens. In addition, PCR/ESI-MS can detect and identify all different pathogens present in polymicrobial samples with quantitative results. This method can also be used on formalin or paraffin embedded tissue (216).

Finally, PCR/ESI-MS has been extensively used for genotyping and serotyping of bacteria and viruses because of its accuracy to detect single nucleotides variations, which represents a tool for health care epidemiological investigation or outbreak follow-up.(23; 62-64; 67; 101; 102; 159; 190; 207; 214; 245; 262). Such an accuracy of the PCR/ESI-MS naturally relies on a representative database and on its maintenance.

Application of PCR/ESI-MS to the diagnosis of BSI directly from blood

The first instrument that was developed was the TIGER (Triangulation Identification for the Genetic Evaluation of Risk) that allowed the detection of specific organisms even in polymicrobial samples (204). The Ibis T5000 instrument, the first commercial version of TIGER could identify up to 800 pathogens from whole blood (65; 261). When compared to blood culture, PCR/ESI-MS applied on 1 ml of whole blood displayed 50% of efficiency. A second commercial version, namely the PLEX-ID (Abbott), using up to 1.25 ml of blood, has been developed displaying good sensitivity and specificity on most of the samples which includes environmental samples as well as clinical samples such as CSF and sputum as said above (15; 38; 76; 91; 138). The first procedure of the PLEX-ID for whole blood consisted of a mechanic cell lysis, using beads, facilitated by the addition of proteinase K, SDS and eating at 56°C.

DNA purification was then achieved with magnetic silica beads and eluted in 250 µl of water at 70°C (137). The PCR is based on 9 pairs of primers for bacteria detection, 4 pairs for *Candida* species and 4 pairs for detection of resistance cassettes (*mecA*, bla_{KPC}, *vanA*, *vanB*). One additional pair of primer corresponds to the extraction control. The PLEX-ID gave 78.6% of agreement with blood culture over 906 specimens taken from adult and pediatric patients (464 with positive blood culture and 442 with negative blood cultures). While 33 culture negative specimens were PLEX-ID positive, 97 culture positive specimens were PLEX-ID negative (137). In this study the estimated sensitivities of PCR/ESI-MS was 85.9% when the estimated sensitivity of BC was 41.2%. To further increase the sensitivity of the detection directly from whole blood a second version of the PLEX-ID that could analyse up to 5 ml of blood has been developed. In this version, the increased sample volume was associated with a DNA extraction method and PCR conditions optimized for whole blood samples (13). Practically, 5 ml of whole blood were lysed in presence of 665 µl of a commercial buffer (100 mM Tris solution containing guanidinium thiocyanate and detergent), 145 µl of BSA 10% containing a pumpkin DNA extraction control), and yttria-stabilized zirconium oxide beads (166). After the removal of lysed red-blood cells by centrifugation the nucleic acid were extracted from the supernatant using silica-coated magnetic beads. Thirty microliters of the eluate were used for the PCR with 25 µl of PCR master mix containing the pool of primers pairs (137). Rather than trying to remove the excess of human DNA, PCR conditions were defined in the context of high concentration of human DNA. This was achieved by testing multiple PCR conditions by modulating Mg^{2+}, primers and polymerase concentration and annealing temperature in the presence of 12 µg of human DNA (13; 73). In this background, high primers concentration (750 µM) together with high polymerase concentration (2.2 units per reaction) resulted in a PCR yield of 86% of the yield when 1 µg human DNA was present. Modulating Mg^{2+} concentration or the annealing temperature had only a weak impact on the PCR yield. This procedure was then evaluated in a prospective study involving 331 patients with suspected BSI (13). For each patient 2 blood bottles (one aerobic and one anaerobic) were inoculated with 5 ml of whole blood and an additional blood sample was collected for PCR/ESI-MS analysis from the same venipuncture (137). The PCR/ESI-MS displayed 83% of sensitivity

and 94% of specificity as compared to BC. Interestingly, this corresponded to 35 positive specimens (10.6%) by PCR/ESI-MS and 18 positive by culture (5.4%). In the absence of any method to investigate the discrepant results, a second aliquot was analysed using PCR/ESI-MS, which confirmed almost all the identifications and increased the sensitivity of PCR/ESI-MS to 91% and the specificity to 99%, were (13). The limit of detection of the PCR/ESI-MS method developed by Bacconi *et al.* is 16 CFU per mL for *S. aureus*, *K. pneumonia*e and *E. faecium*, and 4 CFU per mL for *C. albicans*. It is generally admitted, based on plating methods, that the number of bacteria circulating during BSI is between 1 and 10 CFU per ml (105; 132; 133; 243; 251). The analysis of the data from the literature performed by Bacconi and colleagues estimated that the amount of bacterial DNA in blood during a BSI vary between 10^3 to 10^4 genomes copies per ml (13). This difference could be due to the fact that plating methods reflect the number of cells that survive the procedure rather than the number of circulating cells. Molecular diagnosis directly from blood could detect also free DNA, DNA resulting from dead bacterial cells and bacterial DNA present within phagocytes, which may explain the high performance of the PLEX-ID on whole blood. However, this extremely high sensitivity may lead to contamination by DNA. For this reason, the use of ultra-clean reagents associated with molecular biology laboratory practices is mandatory.

PCR/ESI-MS is also a promising tool for the diagnostic of blood culture negative BSI. Indeed, among 464 patients with positive blood cultures and 442 patients with negative blood culture, 33 culture negative cases were detected positive by PCR/ESI-MS and these cases were demonstrated to be true BSI based on the analysis of the clinical presentation (137). Using primers and probe specific of *Ehrlichia chaffeensis* targeting the 16S gene and a genus specific set of primers and probes Eshoo and colleagues reported 18.8% of PCR/ESI-MS positive specimen of whole blood among 213 blood samples from patients with suspicion of ehrlichiosis (73). From the same pool of specimen, PCR/ESI-MS identified *Rickettsia rickettsii* from 4 samples and *Neisseria meningitidis* from one samples.

PCR/ESI-MS was designed to detect all pathogens present in a mixed microorganism populations (118; 205). This is also true for polymicrobial blood culture since PCR/ESI-MS could identify 29 bottles with

mixed populations out of 234 positive BC bottles (125). A quantitative analysis performed using an internal control helps at (i) interpreting results for polymicrobial samples and (ii) identifying true positive versus contaminating organisms (137). Further studies are required to determine if the quantification could also help at defining the severity of the infection.

In conclusion, the PLEX-ID, which provides reliable results in less than 6 hours, is a versatile system that may be directly used on blood starting from 5 ml of samples and that represents a complementary approach to blood cultures.

MOLECULAR DETECTION OF ANTIMICROBIAL RESISTANCE DURING BLOOD STREAM INFECTIONS

BSI is characterised by a high level of morbidity and mortality that increases with the delay in the introduction of an efficient anti-infectious therapy (58; 82; 90; 113; 148; 149; 157; 232). The rapid identification of the pathogen gives a first indication on the effectiveness of the empiric therapy. However, rapid information on the resistance profile of the agent of a BSI, would allow to better adjust the empirical treatment.

1. Rapid PCR Based-methods for resistance detection from positive blood cultures

Detection of resistance mechanisms of gram-positive bacteria

Staphylococcus aureus is a significant agent of both community-acquired infections and nosocomial infections (256). In recent years, it has been observed an alarming increase of the percentage of methicillin resistant *Staphylococcus aureus* (MRSA) a drug resistant pathogen associated with significant increase of the mortality (49; 50; 169; 170; 263), which explains that many rapid methods are now

available to simultaneously identify *S. aureus* and detect the *mecA* gene associated with methicillin resistant (table 1) (71; 77).

The GeneXpert MRSA/SA BC Assay (Cepheid, Sunnyvale, CA) is a good example of such dual detection method based on real-time PCR for the identification of *S. aureus* and the detection of the *mecA* gene. The analysis may be performed directly from positive BC in about 1 hour and was validated both in adult and pediatric patients with BC positive for gram-negative cocci in cluster with 100% of sensitive and 99.5 to 100 % of specificity (45; 221). The GeneXpert MRSA/SA BC Assay dramatically reduced the time to detection of *S. aureus* and has an impact on the detection of MRSA (figure 2) (45; 54; 140; 208).

Also based on real-time PCR, another rapid PCR-based assay, the StaphSR assay (BD GeneOhm, San Diego, CA), can identify and differentiate methicillin-susceptible *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) from positive blood cultures in about 1.5 h with an analytical sensitivity of 15 DNA copies per reaction mixture which correspond to 10^3 DNA copies per ml (117; 222). The assay was validated on BC with a predominance of gram-negative cocci in cluster showing 95.6% -100% of sensitivity and 95.3% - 98.4% specificity for the detection of MRSA (97; 130; 140; 222). Discrepant results are mainly explained by mixed culture or inhibition of the PCR. Negative MRSA detection is also due to MREJ variants that contain the staphylococcal cassette chromosome *mec* (SCCmec) without the *mecA* gene (219).

The StaphPlex system (Genaco Biomedical Products, Huntsville, AL, USA) is based on multiple PCRs (18 target genes) and microarray analysis for the identification of staphylococci at species-level, for the detection of resistance genes and for the detection of the Panton-Valentine leukocidin (PVL) with a time to result of about 5 hours. The overall accuracy for the detection of staphylococci at the species level from mono-bacterial or poly-bacterial culture was 91.7% when compared to conventional methods (230). The StaphPlex exhibited 100% of sensitivity and 95.5% to 100.0% of specificity for the detection of the *mecA* gene. Similar results were obtained for the detection of the PVL gene (230).

The FilmArray (Idaho Technology, Salt Lake City, UT, USA) is another PCR-based system that can detect the *S. aureus mecA* gene as well as the *vanA* and *vanB* genes implicated in vancomycin resistance in *Enterococcus* species (16; 192). The FilmArray system exhibits a high sensitivity and specificity (100%) for the detection of the *mecA* gene from *S. aureus* positive culture with (16; 19; 196). Another study reported 96% of sensitivity and 98.9% of specificity for the detection of the *mecA* gene (6). For the detection of the *vanA* and *vanB* genes in *Enterococcus spp.*, Blasche *et al.* reported only 85% of specificity of the FilmArray system when compared to BC (19). In this study, the FilmArray detected a *van* gene in 2 *Enterococcus* strains that where found vancomycin sensitive by culture based methods. The second strain was actually an *Enterococcus casseliflavus* strain bearing the *vanC* gene. During the development of the system, more resistance markers have been successfully tested (19).

The microarray of the Verigene gram-negative system (Northbrook, IL, USA) includes probes for the detection of the *mecA* gene with 98.6% of sensitivity and 94.3% of specificity (26). This system also contains probes for the detection of VRE with 100% of sensitivity and specificity in a prospective study from positive BC (26).

The major weakness of the nucleic acid methods is to assign the resistance gene to the correct microorganism in the case of mixed cultures. This is particularly limiting for the detection of the *mecA* gene in a mix culture of *S. aureus* that would contain contaminating coagulase negative staphylococci for instance (26).

Detection of resistance mechanisms of gram-negative bacteria

Gram-negative bacteria can exhibit resistance mechanisms to multiple antibiotics, representing a major problem for the treatment of infections. In particular *Enterobacteriaceae* have developed multiple resistance mechanisms to cephalosporins, such as ESBL. This had presented the carbapenem as alternative for the treatments of severe infections involving *Enterobacteriaceae* and non-fermentative bacteria such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. However, resistance to

carbapenem may also occur. It might be caused by a reduced permeability with loss of porins, by the over-expression of efflux pumps or alternatively by the production of carbapenem degrading enzymes, namely carbapenemases. The emergence of carbapenem resistance strains and their spread worldwide is alarming both from a therapeutic point of view and from an epidemiological point of view. Nucleic acid detection systems are focusing on the bla_{KPC} gene that encode for *K. pneumoniae* carbapenemase. This is the case of the FilmArray system that includes primers targeting the bla_{KPC} gene (6; 17; 19; 196) and the Verigene gram-negative system that contain probes for the detection of the bla_{KPC} (17). More studies involving KPC positive strains are needed to determine the sensitivity and specificity of these systems. Moreover, future tools should aim at detecting a broader range of genes encoding SBL and/or carbapenemases.

2. Resistance detection from positive blood culture using MALDI-TOF MS

The use of the MALDI-TOF MS for the detection of resistant strains was first proposed for the distinction between MRSA and MSSA colonies (68). However, recent developments are mainly directed toward gram-negative bacteria in which The MALDI-TOF MS may detect antibiotics degrading proteins such as carbapenemases. Practically a bacterial inoculum is incubated with ertapenem. The MALDI-TOF MS analysis performed on the supernatant is used to determine the shift of two specific peaks associated with the degradation of the carbapenem. This method has been validated on bacterial colonies with 100% of sensitivity and 100% of specific for the detection of carbapenemase producing strains (240). Carvalhaes and colleagues have proposed a method to be used directly from bacterial pellet obtain from positive BC, which is able to identify 72.4% (21/29) of the carbapenemase-producing isolates after 4 h of incubation (33). MALDI-TOF MS represents an alternative for the detection of carbapenemase from positive BC that has the advantage, over PCR, to provide a phenotypic result.

3. Resistance detection directly from whole blood

As said earlier, the detection of resistance genes directly from blood would considerably impact the choice of the therapy. Four primer set of the PLEX-ID system are used to detect the resistance cassettes *mecA*, *vanA* and *vanB* and bla_{KPC}, (70; 247). It has to be noted that the PLEX-ID system can also detect the presence of the *S. aureus* Panton-Valentine leukocidin toxin (PVL) (247). PCR/ESI-MS was also shown to be successful at identifying the mutations in *gyrA* and *parC* genes involved in *Acinetobacter baumannii* resistance to quinolones (114; 115). In the case of mixed population, quantitative analysis could help at associating a resistance gene to the corresponding pathogen. The MagicPlex system can detect three resistance markers (*mecA*, *vanA*, *vanB*) directly from blood (152). From their study, Carrara *et al*. failed to detect six MRSA strains out of ten using the MagicPlex system (30). The VYOO system can detect five resistance genes: *mecA*, *vanA*, *vanB* β-lactamase bla_{SHV}, β-lactamase $bla_{CTX\text{-}M}$. However there is a lack of studies reporting the sensitivity of this method. Finally, the SeptiFast system contains primers for the detection of *mecA* only (table 1).

Although molecular methods have the ability to detect some resistance genes, more studies are required to determine their performances and their clinical impact. In addition, increasing the number of available resistance markers would be an advantage.

CONCLUSIONS AND FUTURE DIRECTIONS

Nucleic acid methods have succeeded to overcome most of the obstacle that limited the sensitivity of the detection of pathogen from blood containing sample. The sensitivity and specificity are now adequate for the use in diagnostic laboratory.

One major problem for the determination of the performances of new molecular methods is the fact that BC associated with traditional identification methods remains the gold-standard. In this context, many assays display extremely high analytic sensitivity but limited apparent specificity due to a limited correlation with BC. This could be overcome by the use of other evaluation methods to challenge BC. In their study, Bacconi and colleagues showed that 16S sequencing is not an appropriate method to be used as reference for the evaluation of PCR/ESI-MS. Indeed, only 2 of the 35 samples positive using the PCR/ESI-MS were found positive by 16S sequencing when the sequencing was performed on the same material. Thus one of the limits to develop new methods for the diagnostic of BSI is that blood culture remains the gold standard (13). To compare the performance of blood culture and other methods, clinical presentations and local epidemiology should be taken into account in order to properly investigate discrepant results (13; 137). Nevertheless the poor correlation between conventional BC-based methods and some new molecular methods suggest that different pathogens are detected by these two methods and that these diagnostic tools should be complementary. Moreover BC could not be replaced since the availability of a strain in pure culture is mandatory to precisely test antibiotic susceptibility of bacteria.

Although new molecular methods are really appealing, further studies are still needed to determine their impact on the management of patients with BSI (175). For instance, there is a lack of study that addresses the impact on antibiotic stewardship. While most of the studies try to give with precision the performances of the new molecular methods, it is not yet clear how these methods can be integrated in a molecular laboratory. This is particularly true as none of the method would replace BC. Some practical details have to be investigated. Thus, the number of samples, the number of venipuncture and the frequency of the sampling for molecular diagnostic has to be addressed. Otherwise, informations (routinely provided by the BC) on the likelihood of an infection (such as a possible catheter infection) versus a contamination would be missing.

Molecular methods can also be helpful for organisms that are phenotypically closely related or for rare organisms. However, because some genetically closely related organisms cannot be discriminated on the

basis of molecular methods such as *E. coli/Shigella* (59; 210) or group mitis streptococci/*Streptococcus pneumoniae* (126; 224; 227), phenotypic distinction methods remain crucial. In the same manner the accuracy of many molecular methods is based on their databases, which have to be maintained by adding nucleic acid sequences or mass-spectrum obtained for new clinical isolates or rare organisms. For nucleic acid methods, it is also crucial to achieve a follow-up of the emergence of SNP or mutations (insertion/deletion) that could affect the hybridization of specific primers and probes. One solution would be to rely on multiple targets (sequence or gene) for the identification of a given organism (120; 222).

As already stressed earlier the Gram staining remains mandatory for BC based methods since (i) it can help at disclosing poly-microbial infection, (ii) it serves as a quality control and (iii) it sometimes helps identification thanks to specific phenotypic traits. Nevertheless the presence of a single morphotype on the Gram staining, does not exclude the presence of multiple organisms, since *Enterobacteriaceae* often exhibit a similar Gram staining morphotype.

Regarding poly-microbial identification, the quantification provided by some nucleic-acids based methods, can be helpful at determining the significance of the respective organisms. Another research focus is the attempt to provide clinical scores that would predict the severity of bacteremia (78; 195). The time to positivity of blood BC has been proposed as a criterion for BSI severity (7; 113; 129). Similarly, quantification could provide some insight on the severity of the BSI. Thus, analysis of 250 whole-blood samples from 20 adult patients (13 survivors and 7 nonsurvivors) with culture-proven MRSA showed that the levels of *mecA* DNA was higher in the nonsurvivors (5.48 copies/ml) than in the survivors (4.58 log copies/ml P= 0.003, two-tailed Mann-Whitney U test). This suggested that the level of *mecA* DNA in blood could potentially be used to monitor MRSA bacteremia and evaluate responses to therapy (109). Similarly a positive correlation has been found between the level of *Streptococcus pneumoniae* DNA monitored by real-time PCR and the need of mechanical ventilation, the risk of septic shock and of death (32; 198). Another study performed with the SeptiFast on 94 patients reported that the median cycle threshold (Ct) value was 16.9 for patients with severe septic choc and 20.9 for patients with non-severe

sepsis and that Ct value <17.5 correlated with more positive blood culture and longer hospital stays (80; 266).

The quantification can also help at identifying the presence of a contaminant. In this context, the threshold of the quantitative results becomes an important parameter to limit the number of false positive and false negative results. Molecular solutions devoid of quantitative or at least semi-quantitative analysis will render the interpretation of the positive results difficult. The interpretation of poly-microbial detection as well as mono-bacterial ones is facilitated by the fact that PCR/ESI-MS gives is a quantitative analysis performed using an internal control. This can also helps at identifying true positive versus contaminating organisms (137) and at determining the relevance of each pathogen for mixed infections (75). Further studies are required to determine if the quantification could also help at defining the severity of the infection.

Regarding persisting bacteremia in the context of an ongoing treatment, molecular methods have been able to rapidly detect new organisms that BC could not detect. However, nucleic-acid methods are not adequate for the follow-up of persistent infections because they can detect the persistence of DNA from dead organisms rather than true persisting organism. Therefore, many methods are now being developed to detect only DNA from living organisms (1; 172). This could be of particular interest to monitor the efficiency of an antibiotic treatment (29; 248).

Indication on the presence of resistance genes is an important added value of some molecular methods being especially helpful at identifying risk of treatment failure. Development should focus in the integration of more molecular markers. Molecular diagnosis may also detect the presence of virulence genes, which helps predicting the severity of infection. Thus the StaphPlex system helps identifying staphylococci at the species-level and detect resistance genes as well as the Panton-Valentine leukocidin (PVL), excellent sensitivity (100%) and specificity (>95.5%) (230).

In conclusion, molecular diagnosis has significantly improved the diagnosis of BSI due to the reduction of the time to result and to the high sensitivity and specificity. In particular the MALDI-TOF MS is a revolution for the diagnosis of BSI from positive BC. To impact the management of patients suffering of BSIs, microbiologist and clinicians should imagine new laboratories and algorithms that associate these new culture-independent and culture dependant molecular methods with conventional methods in the aim to get the benefits from both diagnosis methods.

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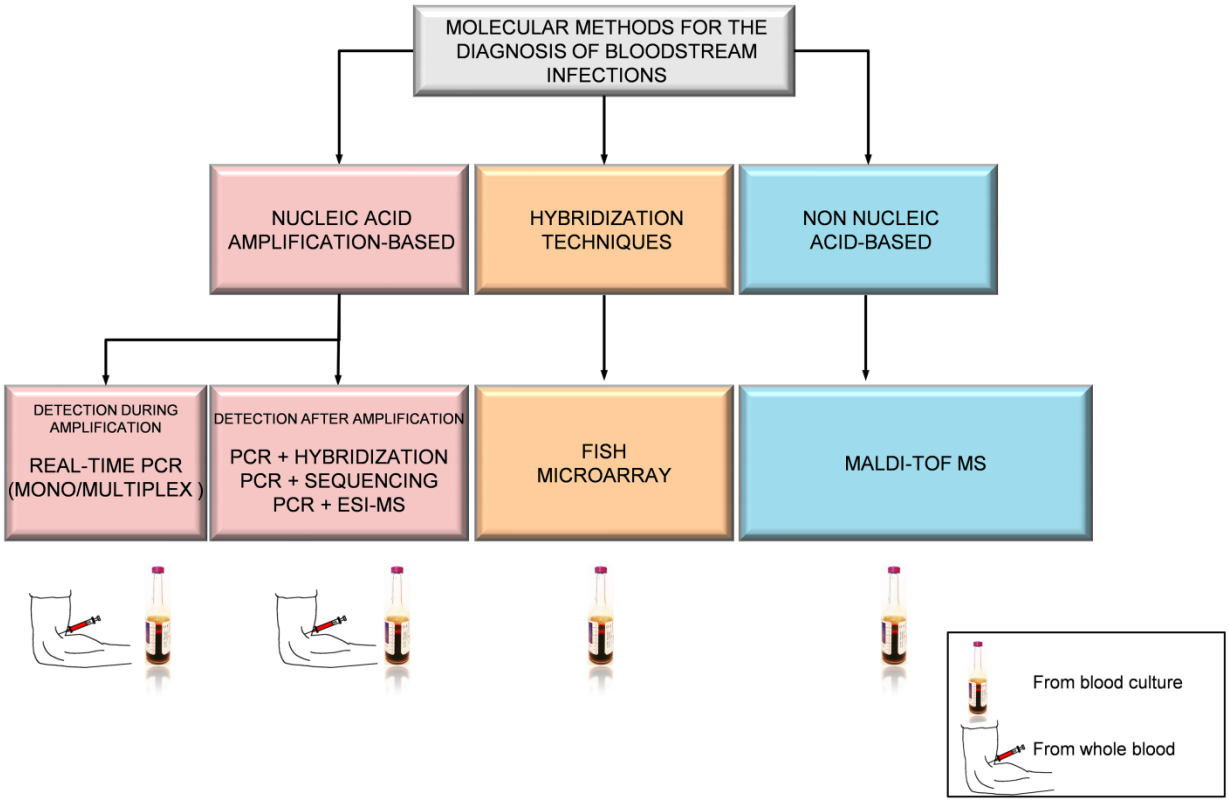
TABLES AND FIGURES

Table 1: Commercially available molecular systems for the microbial identification of pathogen during BSI. Adapted from (174; 175)

SYSTEM (MANUFACTURER)	METHODS	TIME TO RESULT	MICROORGANISM COVERAGE	RESISTANCE AND VIRULENCE MARKERS	SENSITIVITY, SPECIFICITY AND CORRELATION WITH CONVENTIONAL METHODS	REFERENCES
IDENTIFICATION FROM POSITIVE BLOOD-CULTURE						
PNA FISH and QuickFISH (AdvanDx, Wolburn, MA)	FISH	<1-3 hours	4 gram-negative 4 gram-negative 5 fungi	none	97-100% 90-100% 96-99%	(31; 55; 56; 85; 103; 106; 167; 220; 225)
AccuProbe (Gen-Probe, San Diego, CA, USA)	FISH	<1 hour	*S. aureus* *Enterococcus* spp. *S. pneumoniae* *Streptococcus* group A *Streptococcus* group B	none	80.8-100% 98.7-100% nr	(9; 151)
Verigene (Nanosphere, Northbrook, IL, USA)	Microarray	2.5 hours	12 gram-negative 9 gram-negative	*mecA*, *van*A/B, bla_{KPC}, bla_{NDM}, CTX-M,VIM,IMP,OXA12	81-100% 98-100% nr	(5; 14; 17; 26; 60; 155; 165; 226; 227; 234)
Prove-it Sepsis (Mobidiag, Finland)	Microarray	3.5 hours	60 bacteria 13 fungi	*mecA*	95 % 99 % nr	(233)
FilmArray (Idaho Technology, Salt Lake City, UT, USA)	Multiplex PCR	1 hour	8 gram-negative 11 gram-negative 5 fungi	*mecA*, *van*A/B, bla_{KPC}	97-95% 91-98% nr	(6; 19; 181; 196)
Xpert MRSA/SA BC (Cepheid, Sunnyvale, CA, USA)	Real-time PCR	1 hour	*S. aureus*	*mecA*	100% 99-100% nr	(45; 54; 140; 208; 221)
StaphSR assay (BD GeneOhm, San Diego, CA, USA)	Multiplex PCR	1-2 hours	*S. aureus*	*mecA*	96-100% 95-98% nr	(97; 130)
StaphPlex (Genaco Biomedical Products, Huntsville, AL, USA)	Multiplex PCR + Microarray	5 hours	*S. aureus*	*mecA* (+ PVL)	100% 95-100% 92%	(230)
MALDI-TOF MS Bruker Daltonics (Bremen, Germany) or bioMérieux (Marcy l'Etoile, France)	Mass-spectrometry	<1 hour	<1000[a]	not in routine	- - 76-99%	(42; 52; 156; 158; 193; 194; 224)
IDENTIFICATION FROM WHOLE BLOOD						
SepsiTest (Molzym, Bremen, Germany)	Broad range PCR + sequencing	6 hours (1-10[b] ml)	>345 bacteria and fungi	none	21-87% 85-96% nr	(145; 152; 177; 200; 212; 250)
SeptiFast (Roche Molecular System, Germany)	Multiple broad-range real time PCR	3.5-5 hours (1.5 ml)	6 Gram positive 8 gram-negative 5 fungi	*mecA* [b]	43-95% 60-100% 43-83%	(25; 35; 53; 80; 95; 124; 127; 139; 143; 145-147; 153; 154; 161; 164; 182; 186; 197; 244)
MagicPlex (Seegene, Seoul, Korea)	Multiple PCR+ multiplex real time PCR	3-5 hours (1ml)	21 bacteria at species level (90 at genus level) 6 fungi	*mecA, vanA/B*	37-65% 77-92% 73%	(30; 152)
VYOO (SIRS-Lab, Jena, Germany)	Multiplex PCR + electrophoresis	8 hours (5ml)	14 Gram positive 18 gram-negative 7 fungi	none	nr nr 70%	(20; 84; 212)
PLEX-ID (Abbott Molecular, Des Plaines, IL)Laboratories, IL, USA)	Multiplex broad-range PCR + ESI-MS	6 hours (1.25-5[c] ml)	up to 800 (Gram positive, Gram negative, fungi)	*mecA*, bla_{KPC}, *vanA/B*	50-91%[d] 98-99% 79-97%	(13; 166)

[a] Dependant on the mass-spectrum database
[b] With an additional kit
[c] For the latest version
[d] depending on the volume
nr=non reported



1752

1753 **Figure 1: Different molecular technologies used for the detection and identification of microbes**
1754 **during bloodstream infections.** Molecular methods for the diagnosis of BSI include nucleic acid-based
1755 methods and non-nucleic acid-based. Nucleic acid amplification-based techniques can be applied on
1756 positive blood cultures or used directly on blood whereas non nucleic acid amplification-based techniques
1757 such as FISH (fluorescent in situ hybridization) and microarray or non-nucleic acid based methods such
1758 as MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry)
1759 can be used only on positive blood culture. Adapted from (150).

1760

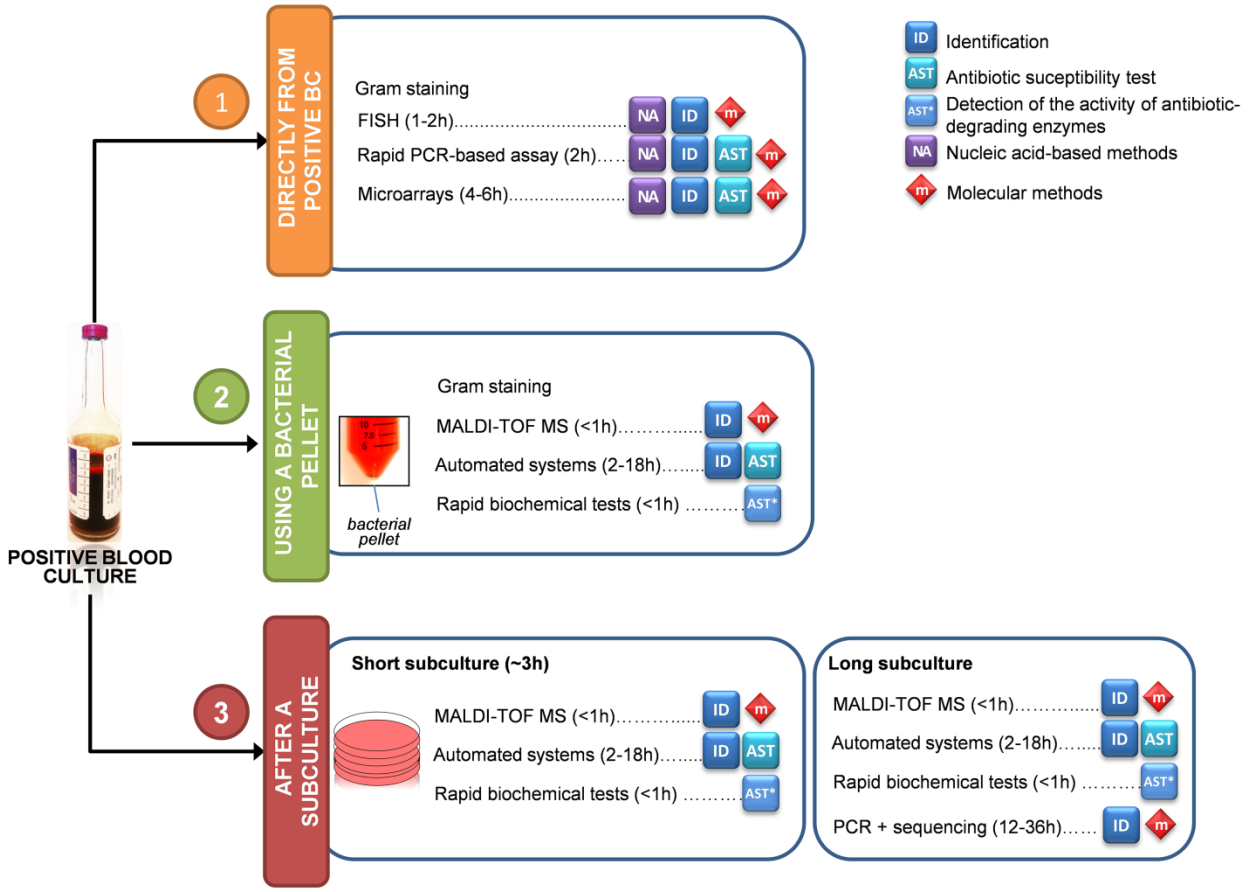


Figure 2: Conventional and molecular methods for the identification of microorganisms from positive blood culture. When a blood culture is detected as positive several strategies are available to identify the incriminated microorganism after the initial Gram staining: (1) Identification directly from the positive blood culture using nucleic acid-based methods, (2) Identification after microbe's enrichment, namely a purified bacterial pellet suitable for MALDI-TOF MS analysis and some automated identification/AST approaches such as Vitek2 and Phoenix and rapid biochemical tests HMRZ and ESBLNP (3) Identification after a subculture; to date the automated system Vitek2 has been validated on short subculture as well as the rapid biochemical tests HMRZ and CARNP; long subculture are suitable for any type of analysis including phenotypic characterization, automated systems such as Vitek2, Phoenix and MicroScan WalkAway as well as PCR followed by sequencing. Adapted from (174).

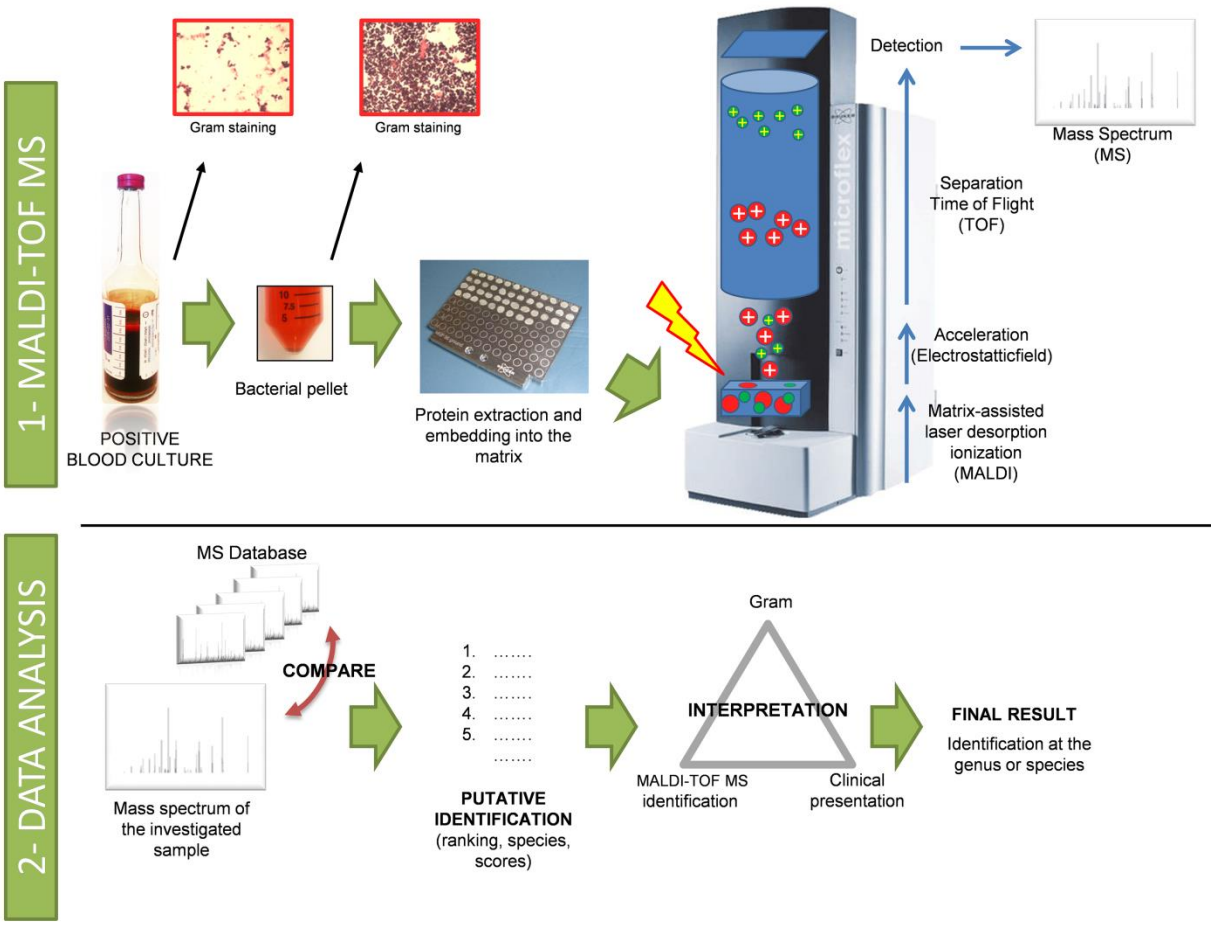


Figure 3: Microbial identification from positive blood culture using MALDI-TOF MS. The sample that is deposited on the MALDI-TOF multi-well plate can be a bacterial pellet obtained by centrifugation and erythrocytes lysis. Alternatively, the sample can be bacteria obtained after a subculture. Proteins are then extracted and embedded in a matrix directly on the multi-well conductive metal plate and submitted to the MALDI-TOF MS, which separate the proteins according to their MW and their charges. This generates a mass spectrum (MS) which, in a second stage is compared to a database of spectra. This analysis provides the identification of the microorganism with a confidence score, which allows acceptation at the species or at the genus level. The identification is interpreted according to the Gram staining and to the clinical presentation. Adapted from (52).

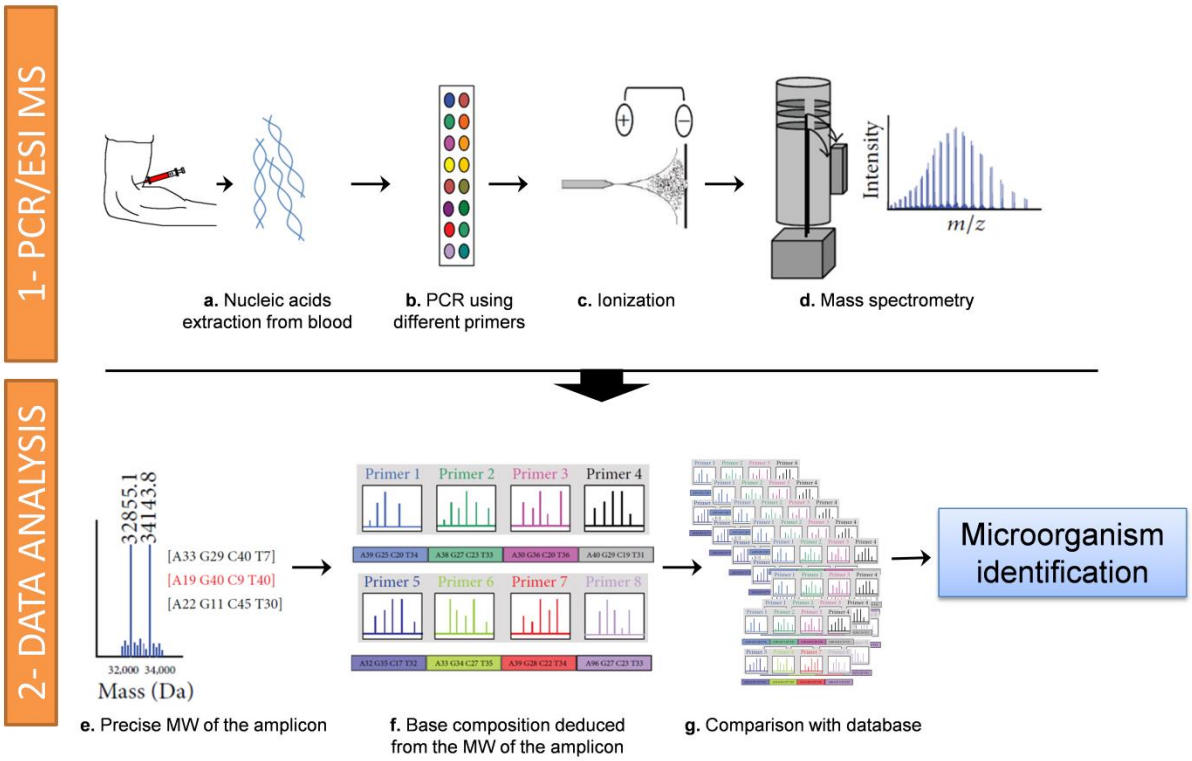


Figure 4: Schematic workflow of PCR/ESI-MS. (a) Nucleic-acids are extracted from the sample, directly from whole blood, and **(b)** amplified by multiple PCR using multiple pairs of primers; each colour represents a different primer. After amplification, the molecular mass of the amplicon(s) is precisely determined using ESI-MS **(c, d and e),** from which **(f)** the base composition of the amplicon(s) is deduced. Finally, **(g)** informations - base composition - obtained from one or more amplicon(s) are compared with a database, which provides the identification with a confidence score. Adapted from (123).