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1 **“Molecular and Mass Spectrometry Detection and**  
2 **Identification of Causative Agents of Bloodstream**  
3 **Infections”**

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54 **ABSTRACT**

55

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

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## 77 INTRODUCTION

78

### 79 1. Bloodstream infections

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

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

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

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

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

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

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

## 133 **2. Blood culture-based diagnosis of bloodstream infections**

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



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

152 Inside positive BC, bacteria concentration reaches  $1.10^6$  to  $2.10^8$  for gram-negative cocci and  $2.10^7$  to  
153  $1.10^9$  for gram-negative bacilli (42; 229). These concentrations allow the use of amplification-based  
154 methods such as PCR and real-time PCR as well as non amplification-based methods such hybridization  
155 or MALDI-TOF MS (figure 1).

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

### 165 **3. Microbial diagnosis of bloodstream infections directly from blood**

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

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

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

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

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

## 203 **MOLECULAR METHODS FOR THE IDENTIFICATION OF PATHOGEN** 204 **FROM POSITIVE BLOOD CULTURES**

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

## 219 1. Fluorescent in situ hybridization (FISH)

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

233 The Quick-FISH system is a more rapid version of the PNA-FISH system. The turnaround time (TAT) is  
234 shortened by reducing the hybridization phases to 15 min and by removing the wash step. At present, the  
235 QuickFISH system provides kits that detect and distinguish *S. aureus*/Coagulase negative staphylococci,  
236 *Enterococcus faecalis*/*Enterococcus faecium*, *E. coli*/*P. aeruginosa* (55; 56; 158). The diversity of  
237 microorganisms that is currently detected is still limited but these bacterial species represent about 95% of  
238 the pathogen involved in BSI. The choice of the probe to be tested will depend on the Gram result, the  
239 clinical presentation and the local epidemiology. The sensitivity of the QuickFISH system is 99.5% for  
240 the detection of *S. aureus* and 98.8% for coagulase negative staphylococci, with a combined specificity of  
241 89.5% (55). Martinez et al. reported 97.9% of concordance with conventional detection methods for  
242 Gram-positive bacteria and 95.7% for gram-negative bacteria (158). Yeast (*C. albicans*, *C. parapsilosis*,

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

## 247 **2. Microarray**

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

267 from positive BC between the Verigene Gram-Positive Blood Culture system and the traditional method  
268 (5; 14; 158; 165; 206; 227; 258). Misidentifications have been reported for *Streptococcus* spp.. In  
269 particular group mitis *Streptococcus* spp. have been misidentified with *S. pneumoniae* and *S. oralis* have  
270 been misidentified with *S. anginosus* (206). Performances of the Verigene system are higher on mono-  
271 bacterial samples. Mixed culture can prevent the identification of one of the pathogen. Buchan et al  
272 reported 98.6% of sensitivity and 94.3% of specificity for the detection of the *mecA* gene in 5999  
273 *Staphylococci* cultures, and 100% of sensitivity and specificity for the detection the *vanA* gene in 81  
274 cultures containing *Enterococcus faecalis* and *Enterococcus faecium* (26). Samuel et al reported 91% of  
275 concordance for *mecA* and 100% for *vanA* detection when compared to routine methods (206). In case of  
276 mixed population of staphylococci, the resistance gene cannot be associated to a specific pathogen (26).  
277 The Verigene Gram-Negative can detect 5 pathogens at the species level (*Escherichia coli*, *Klebsiella*  
278 *pneumoniae*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa* and *Serratia marcescens*) and 4 genus  
279 (*Acinetobacter* spp., *Citrobacter* spp., *Enterobacter* spp. and *Proteus* spp.). The Verigene Gram Negative  
280 BC assay displays 91 to 100% of agreement for the detection of gram negative pathogens both from adult  
281 and pediatric patients (17; 60; 108; 155; 226). Among, the false-negative results, mixed blood cultures  
282 have been incriminated; but some of these negative or non called results involved bacteria covered by the  
283 chip (60). As for many molecular assays, it is not possible to distinguish *E. coli* and *Shigella* spp. (*S.*  
284 *dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*). This distinction should be achieved with enzymatic  
285 analysis. The Verigene Gram Negative assay can detect 6 resistance genes: the extended-spectrum beta-  
286 lactamase (ESBL) CTX-M and the carbapenemases KPC, NDM, VIM, IMP and OXA groups with 92.3%  
287 to 100% of concordance with routine methods (60; 234). Other microarrays for the detection of resistance  
288 gene are currently being developed (24).

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

### 291 3. Nucleic acid amplification-based methods

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

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

#### 308 Multiplex PCR

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

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

315 In a prospective study on 102 blood cultures, the FilmArray system displayed 91% of sensitivity and 77%  
316 of specificity when compared to conventional identification methods for the detection of pathogen present  
317 in the FilmArray panel only (19). The detection of resistance genes revealed 100% of specificity and  
318 100% of sensitivity in this study. In another study, FilmArray displayed 98.5% of sensitivity and 100% of  
319 specificity for the identification of gram-negative bacteria, 96.7% of sensitivity and 93.7% of specificity  
320 form gram-negative bacteria and 96% of sensitivity and 98.9% of specificity for the detection of the *mecA*  
321 gene. Another study performed on 118 mono-bacterial culture reported 92% of correct identification of  
322 the FilmArray system, but mixed cultures gave no results (17). The identification of *Candida albicans*  
323 and *Candida glabrata* reached 100% of sensitivity and 99.5% of specificity (6). This is consistent with  
324 other study showing high performance of the FilmArray for the identification *C. albicans* and *C. glabrata*  
325 (181). The FilmArray can also be applied on other sterile samples such as CSF (184).

### 326 **Rapid PCR-based systems**

327 The clinical and epidemiological impact of pathogens such as methicillin resistant *Staphylococcus aureus*  
328 (MRSA) or rifampicin resistance *Mycobacterium tuberculosis* has contributed to the development of  
329 methods for the rapid detection of resistance. *S. aureus* is a significant agent of community-acquired and  
330 nosocomial infections (256). The mortality rate is increased in patient infected with MRSA in comparison  
331 to patient infected with MSSA (methicillin sensitive *S. aureus*) (49; 50; 263). The increased percentage of  
332 MRSA in comparison to MSSA has stressed the importance of rapid detections methods of MRSA (71;  
333 77; 169; 170). Several methods based on multiplex real-time PCR are available: the GeneXpert  
334 MRSA/SA BC Assay (Cepheid, Sunnyvale, CA) (45; 221), the StaphSR assay (BD GeneOhm, San  
335 Diego, CA) (117; 222) and the StaphPlex (Genaco Biomedical Products, Huntsville, AL, USA) (230).  
336 The GeneXpert and StaphSR assay are rapid PCR-based systems developed for the detection of *S. aureus*



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

#### 345 **4. Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass** 346 **Spectrometry (MALDI-TOF MS)**

##### 347 **Principle of MALDI-TOF MS**

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

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

360 identification has been achieved directly from whole bacteria coming from single colonies (44; 110).  
361 Identification of bacteria using the MALDI-TOF MS is divided in three steps: (1) ionization of the  
362 sample, (2) separation of proteins in a flight tube (3) generation of a mass spectrum by determination of  
363 the mass of the proteins (figure 3). The sample preparation consists in mixing the cells from a bacterial  
364 colony, with a crystallizing matrix that will trigger the ionization of the sample achieved by a laser  
365 (MALDI). Protein accessibility can be facilitated by a quick formic acid extraction. Ionized proteins are  
366 accelerated by an electrostatic field and separated along a flight tube. The separation is due to the time of  
367 flight (TOF), a function of the mass and of the charge of the proteins. Protein detections will generate a  
368 mass spectrum unique for a defined bacterial strain corresponding to mass-to-charge ratio ( $m/z$ ) between  
369 1000 and 20000 kDa. However, several peaks are shared between bacteria from the same genus or species  
370 and serve as biomarkers for bacterial identification. The identification is performed by comparison of the  
371 mass spectrum with a database of spectra obtained from characterized bacterial strains. The software that  
372 performed the spectral analysis and the comparison with the database, assign a score for the identification.  
373 Depending on the threshold recommended by the furnisher and the in-house algorithm, the identification  
374 can be rejected, or accepted either at the species level or only at the genus level.

### 375 **Application of MALDI-TOF MS on positive blood culture bottle**

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

384 *Application of MALDI-TOF MS after a short subculture*

385 A short subculture (2 to 3 hours) on agar plates can be performed from the positive blood vial to obtain a  
386 thin layer of bacteria (174). From this thin layer, the MALDI-TOF MS analysis can be performed (figure  
387 2) (116; 239). Short subcultures are also adequate for AST using automated systems (116). However, this  
388 procedure is mainly suitable for fast growing bacteria.

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

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

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

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

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

#### 415 *Efficiency and reproducibility of MALDI-TOF MS identification*

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

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

429 *Importance of the Gram staining and of the subculture*

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

435

436 **MOLECULAR METHODS FOR THE DETECTION OF PATHOGENS**

437 **DIRECTLY FROM BLOOD**

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

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

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

### 453 **1. Broad range PCR**

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

460 The 16S rRNA gene that displays sufficient level of conservation among bacterial species is the target of  
461 in-house PCRs used in many molecular diagnostic laboratories (12; 40; 41; 43; 94). In many organisms,  
462 the ribosomal operon is present in multiple copies which increases the sensitivity of PCRs targeting this  
463 genomic region. The intergenic spacer is more polymorphic and more species specific. Specific PCR  
464 primers are designed on conserved regions that surround highly variable region. Thus, the sequencing of  
465 the amplicon allows the identification of the microbe at the genus or at species level. Most of these PCR  
466 cannot be used directly on blood because of their limited sensitivity and specificity. However, some  
467 studies have reported sensitivity of PCR targeting the 16S rRNA gene ranging from 10 to  $2.5 \cdot 10^2$  CFU  
468 per reaction directly from blood (131; 191). Gaibani *et al* have developed a broad-range real-time PCR  
469 that targets a 97 base pair sequence of the 23S rRNA gene. This PCR is expected to detect 90% of the  
470 bacteria involved in BSI but does not give any identification, since the short fragments amplified do not  
471 allow discrimination upon sequencing (89). This real-time PCR can be used on whole blood (100  $\mu$ l). Its  
472 sensitivity is ranging from 10 to  $10^3$  CFU per reactions for *E. coli* and *S. aureus*, depending on the  
473 extraction method.

474 The SepsiT<sub>est</sub> system (Molz<sub>ym</sub>, Bremen, Germany) is based on broad-range PCR using universal primers  
475 that target the 16S rRNA gene for bacterial identification and the 18S rRNA for fungal identification. The  
476 amplicon are sequenced and analysed by BLAST to give identification at the species level (=> 99% of  
477 identity) or genus level (=> 97%) (134; 250). Of course this cut-off does not apply to all bacterial species  
478 and should be used with caution. The analysis is achieved from 1ml of whole blood. After lysis of human  
479 cells, the human DNA is degraded by a DNase. The PCR is achieved using reagent provided by the  
480 manufacturer and lead to amplicon of about 450 base pairs. The time to result is approximately 6 hours.  
481 Depending on the study, the sensitivity and specificity are variable when compared to BC. A first study  
482 performed in critically-ill patients led to 28.6% of sensitivity and 85.3% of specificity of the SepsiT<sub>est</sub>  
483 when BC is used as gold standard (145; 212). In a second study, pathogen could be detected in 26 %  
484 (13/50) of the critically-ill patients while SepsiT<sub>est</sub> could detect pathogens in only 12 % of the patients  
485 (212). In a third study, still using BC as gold standard, SepsiT<sub>est</sub> sensitivity was 21% and specificity was  
486 96% (152). In contrast, high sensitivity and acceptable specificity (87% and 85.5%) was monitored in a  
487 multicenter study involving 342 blood samples from 187 patients (250). In a study performed on patients  
488 supported by extracorporeal membrane oxygenation, the sensitivity and specificity were 78.6 % and 88.4  
489 % when compared to BC with 97.7% of concordance of positive results (177; 200). In this study,  
490 SepsiT<sub>est</sub> could detect at least a pathogen in 25% of patients with negative BC. For patient with suspected  
491 endocarditis, Kuhn *et al.* reported higher performances of SepsiT<sub>est</sub> over BC (134). The variable  
492 sensitivity and specificity of the SepsiT<sub>est</sub> when compared to BC needs to be investigated. One specific  
493 feature of SepsiT<sub>est</sub> is that it can be used on sterile samples other than blood with the potential to replace  
494 home-made broad-range PCR (96; 200). A limitation of this method is that the extraction is not automated  
495 (99). Another important limitation of all these molecular tests is that they do not provide strain for  
496 detailed antibiotic susceptibility testing.

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

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

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

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

## 518 **2. Real-time PCR and Multiplex PCR**

519

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



523 *Principle of the LightCycler SeptiFast system*

524 The LightCycler SeptiFast system is based on broad-range real-time PCR that can identify 19 pathogens  
525 (8 gram-negative bacteria, 6 gram-negative bacteria and 5 fungi) representing approximately 90% of the  
526 pathogen responsible for BSI. The SeptiFast can be used directly from whole blood with an overall time  
527 to result of about 5 hours (3.5 when associated to an automated extraction system). The targets of the  
528 multiplexed broad-range real-time PCR are the internal transcribed spacer (ITS) between the 16S and the  
529 23S ribosomal DNA for bacteria and the ITS between the 18S and the 5.8S ribosomal DNA for fungi.  
530 Identification at the species level is determined by the distinct melting curves of the specific probes. The  
531 LightCycler can detect 8 gram-negative bacteria detected: *E. coli*, *Klebsiella pneumoniae*/ *Klebsiella*  
532 *oxytoca*, *Enterobacter cloacae*/ *Enterobacter aerogenes* species, *Proteus mirabilis*, *Pseudomonas*  
533 *aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*. Six Gram-positive bacteria can be  
534 recognized: *S. aureus*, coagulase negative staphylococci, *Streptococcus pneumoniae*, *Streptococcus*  
535 *pyogenes*, *Streptococcus agalactiae* / *Streptococcus mitis*, *Enterococcus faecium* and *Enterococcus*  
536 *faecalis*. Six fungi can be detected and identified at the species level: *Candida albicans*, *Candida*  
537 *tropicalis*, *Candida parapsilosis*, *Candida glabrata*, *Candida krusei* and *Aspergillus fumigatus*. Basically,  
538 the assay consists of three separated reactions that contain distinct primers and probes mixes to detect  
539 respectively gram-negative bacteria, gram-negative bacteria and fungi. The LightCycler SeptiFast is  
540 designed for the analysis of 1.5 ml of blood on which a mechanic cell lysis is achieved with the SeptiFast  
541 Lys KIT MGRADE and the MagNALyser from Roche diagnostic prior DNA extraction using also a  
542 commercial kit provided by the same manufacturer. The assay contains an internal control that consist of  
543 a synthetic double-strand DNA, similar to the expected amplicon but with a distinct probe binding site.

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

547 *Diagnosis of Infection in febrile neutropenia using the LightCycler SeptiFast*

548 From a group of 86 febrile neutropenic patients representing 141 episodes of fever, BC and SeptiFast  
549 detected approximately the same number of microorganisms: 44/141 (31.2 %) and 42/141 (29.8%),  
550 respectively. However, the association of BC and SeptiFast increases the rate of documentation from  
551 about 30% to 43% (61/141) which might be due to the fact that only 12 organisms were detected by both  
552 BC and SeptiFast (139). A similar observation was made by Mancini *et al.* on a study performed on 103  
553 samples from neutropenic patient with haematological diseases in which 20.4% of the samples were  
554 positive with BC and 33% using SeptiFast, with only 83% of correlation between the two methods (154).  
555 For Bravo *et al.*, the agreement between BC and SeptiFast was 69% for neutropenic patients and 75% for  
556 patients from the intensive care unit (25). All these studies suggest that SeptiFast cannot replace BC but  
557 that the two techniques could be complementary (98; 139; 154; 161). Lamoth and colleagues suggested  
558 that the low sensitivity of SeptiFast was due i) to organisms absent from the SeptiFast analytic spectrum  
559 (40% of false negative) and ii) to the cut-off that decrease the sensitivity for the detection of coagulase  
560 negative staphylococci or for streptococci and increased the rate of false negative (139). False negative  
561 results obtained for gram-negative cocci might also be explained by the inefficient lysis of these  
562 microorganisms (182) or to PCR inhibition in the case of high bacterial load. The adjustment of the  
563 experimental procedure and an adequate cut-off might increase the performance of the SeptiFast assay for  
564 neutropenic patients (139; 182). In a context of persistent fever, SeptiFast identified new pathogens in  
565 89% of the cases whereas BC identifies 8% of the pathogens (139). Similar results were obtained by von  
566 Lilienfeld-Toal *et al.* (241). In this study all the patients with a probable fungal infection had positive  
567 SeptiFast results for *Aspergillus fumigatus*. This suggests a potential added value of the SeptiFast assay  
568 for the detection of fungemia in neutropenic patients (35; 139; 154; 180; 241).

569 *Diagnosis of infectious endocarditis*

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

581 *Diagnosis of neonatal sepsis*

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

587 *Advantages and inconvenient of the LightCycler SeptiFast system*

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

592 (<5hours). In a study performed on 114 consecutive patients with clinical evidence of sepsis the mean  
593 time to results for SeptiFast was less than 8 hours when BC mean time to positive result was 3.5 days and  
594 for negative result 5 days (218). In a study performed on patients of the emergency department with  
595 suspected sepsis, Schaub *et al.* Reported a median time to positivity of BC of 16 hours (without the  
596 organism identification) (209). SeptiFast might be useful in case of a persistent bacteremia, for instance in  
597 neutropenic patient with persistent fever, for which SeptiFast often identified additional pathogens. There  
598 are a high number of studies on the performance of the SeptiFast. However, there is a need of  
599 interventional studies to clearly determine the clinical impact of this new technology.

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

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

615 *The MagicPlex Sepsis system*

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

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

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

635

636

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

#### 638 **Principle, characteristics and performance of PCR/ESI-MS**

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

646 The PCR/ESI-MS approach is based (i) on the amplification of microorganisms DNA by multiple PCRs  
647 and (ii) on the identification of the organism(s) at the species or genus level through the analysis of the  
648 amplicon by ESI-MS (Electrospray Ionization Mass Spectrometry) (65; 123). Basically, PCR/ESI-MS  
649 consists in 5 steps: 1) extraction of the microorganism or sample DNA, 2) amplification of the DNA  
650 using multiple pairs of primers, 3) precise determination of the molecular mass of the amplicon(s) using  
651 ESI-MS, 4) deduction of the base composition of the amplicon(s) from the exact MW of the amplicon 5)  
652 identification of the pathogen(s) by integrating the informations obtained from several amplicon(s) (figure  
653 4) (66; 123).

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

660 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  
661 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  
662 extension of 2 min at 72°C and hold at 4°C hold (73). Major innovation of PCR/ESI-MS is that the  
663 pathogen's identification relies only on the base composition (A, C, G and T) of the amplicons (65; 123).  
664 The base composition is obtained by the integration of the exact mass of the amplicon, the length of the  
665 amplicon, the mass of each base and the complementarily rules of DNA (figure 4). The choice of the  
666 PCR targets makes the base composition of one or more amplicon(s) sufficient for pathogen  
667 identification. The absence of Sanger sequencing dramatically reduces the time to result(s). Thus, ESI-MS  
668 provides the base composition of an amplicon in about 1 minute, the mass of the amplicon being  
669 determined by the time of flight (TOF). The base composition of the amplicon(s) is compared to a  
670 database providing the identification at the genus or at the species level with a score of probability (Q-  
671 score) inferred by an ESI-MS triangulation software relying on multiple amplified regions. The Q-score  
672 integrates multiples parameters such as the number of primer pairs that gave an amplicon, the number of  
673 potential microorganism, the proximity of the base compositions to reference matches in the database.

674 A set of 9 pairs of primers is used for the coverage of the bacteria kingdom. Four pairs are necessary for  
675 *Candida* species. Four additional pairs of primers have been designed to detect the resistance cassettes  
676 *mecA*, *bla<sub>KPC</sub>*, *vanA* and *vanB*. Using clinical samples (up to 1.25 ml) other than blood, PCR/ESI-MS  
677 displays good sensitivity and specificity. This includes environmental samples as well as clinical samples  
678 such as CSF (75; 76; 171) and respiratory tract samples (64; 91; 119). Compared to culture the PCR/ESI-  
679 MS correctly identified 95.6% and 81.3% of the strains at genus level and species levels respectively.  
680 Among 395 respiratory samples, PCR/ESI-MS displayed 67.6% of agreement at the genus level and  
681 66.6% at the species level with culture. PCR/ESI-MS was able to identify fungi in 20.3% (35/172) of the  
682 respiratory specimens with a negative culture suggesting a better sensitivity than culture (217). A  
683 commercial kit available for the detection of viruses (38; 39; 48; 107; 135; 142; 185; 228; 231) has been  
684 shown to display higher performance than real-time PCR and microarrays (228). PCR/ESI-MS have also

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

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

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

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

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



709 DNA purification was then achieved with magnetic silica beads and eluted in 250 µl of water at 70°C  
710 (137). The PCR is based on 9 pairs of primers for bacteria detection, 4 pairs for *Candida* species and 4  
711 pairs for detection of resistance cassettes (*mecA*, *bla<sub>KPC</sub>*, *vanA*, *vanB*). One additional pair of primer  
712 corresponds to the extraction control. The PLEX-ID gave 78.6% of agreement with blood culture over  
713 906 specimens taken from adult and pediatric patients (464 with positive blood culture and 442 with  
714 negative blood cultures). While 33 culture negative specimens were PLEX-ID positive, 97 culture  
715 positive specimens were PLEX-ID negative (137). In this study the estimated sensitivities of PCR/ESI-  
716 MS was 85.9% when the estimated sensitivity of BC was 41.2%. To further increase the sensitivity of the  
717 detection directly from whole blood a second version of the PLEX-ID that could analyse up to 5 ml of  
718 blood has been developed. In this version, the increased sample volume was associated with a DNA  
719 extraction method and PCR conditions optimized for whole blood samples (13). Practically, 5 ml of  
720 whole blood were lysed in presence of 665 µl of a commercial buffer (100 mM Tris solution containing  
721 guanidinium thiocyanate and detergent), 145 µl of BSA 10% containing a pumpkin DNA extraction  
722 control), and yttria-stabilized zirconium oxide beads (166). After the removal of lysed red-blood cells by  
723 centrifugation the nucleic acid were extracted from the supernatant using silica-coated magnetic beads.  
724 Thirty microliters of the eluate were used for the PCR with 25 µl of PCR master mix containing the pool  
725 of primers pairs (137). Rather than trying to remove the excess of human DNA, PCR conditions were  
726 defined in the context of high concentration of human DNA. This was achieved by testing multiple PCR  
727 conditions by modulating Mg<sup>2+</sup>, primers and polymerase concentration and annealing temperature in the  
728 presence of 12 µg of human DNA (13; 73). In this background, high primers concentration (750 µM)  
729 together with high polymerase concentration (2.2 units per reaction) resulted in a PCR yield of 86% of the  
730 yield when 1 µg human DNA was present. Modulating Mg<sup>2+</sup> concentration or the annealing temperature  
731 had only a weak impact on the PCR yield. This procedure was then evaluated in a prospective study  
732 involving 331 patients with suspected BSI (13). For each patient 2 blood bottles (one aerobic and one  
733 anaerobic) were inoculated with 5 ml of whole blood and an additional blood sample was collected for  
734 PCR/ESI-MS analysis from the same venipuncture (137). The PCR/ESI-MS displayed 83% of sensitivity

735 and 94% of specificity as compared to BC. Interestingly, this corresponded to 35 positive specimens  
736 (10.6%) by PCR/ESI-MS and 18 positive by culture (5.4%). In the absence of any method to investigate  
737 the discrepant results, a second aliquot was analysed using PCR/ESI-MS, which confirmed almost all the  
738 identifications and increased the sensitivity of PCR/ESI-MS to 91% and the specificity to 99%, were (13).  
739 The limit of detection of the PCR/ESI-MS method developed by Bacconi *et al.* is 16 CFU per mL for *S.*  
740 *aureus*, *K. pneumoniae* and *E. faecium*, and 4 CFU per mL for *C. albicans*. It is generally admitted, based  
741 on plating methods, that the number of bacteria circulating during BSI is between 1 and 10 CFU per ml  
742 (105; 132; 133; 243; 251). The analysis of the data from the literature performed by Bacconi and  
743 colleagues estimated that the amount of bacterial DNA in blood during a BSI vary between  $10^3$  to  $10^4$   
744 genomes copies per ml (13). This difference could be due to the fact that plating methods reflect the  
745 number of cells that survive the procedure rather than the number of circulating cells. Molecular diagnosis  
746 directly from blood could detect also free DNA, DNA resulting from dead bacterial cells and bacterial  
747 DNA present within phagocytes, which may explain the high performance of the PLEX-ID on whole  
748 blood. However, this extremely high sensitivity may lead to contamination by DNA. For this reason, the  
749 use of ultra-clean reagents associated with molecular biology laboratory practices is mandatory.

750 PCR/ESI-MS is also a promising tool for the diagnostic of blood culture negative BSI. Indeed, among  
751 464 patients with positive blood cultures and 442 patients with negative blood culture, 33 culture negative  
752 cases were detected positive by PCR/ESI-MS and these cases were demonstrated to be true BSI based on  
753 the analysis of the clinical presentation (137). Using primers and probe specific of *Ehrlichia chaffeensis*  
754 targeting the 16S gene and a genus specific set of primers and probes Eshoo and colleagues reported  
755 18.8% of PCR/ESI-MS positive specimen of whole blood among 213 blood samples from patients with  
756 suspicion of ehrlichiosis (73). From the same pool of specimen, PCR/ESI-MS identified *Rickettsia*  
757 *rickettsii* from 4 samples and *Neisseria meningitidis* from one samples.

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

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

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

## 767 **MOLECULAR DETECTION OF ANTIMICROBIAL RESISTANCE** 768 **DURING BLOOD STREAM INFECTIONS**

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

### 774 **1. Rapid PCR Based-methods for resistance detection from positive blood** 775 **cultures**

#### 776 **Detection of resistance mechanisms of gram-positive bacteria**

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

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

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

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

798 The StaphPlex system (Genaco Biomedical Products, Huntsville, AL, USA) is based on multiple PCRs  
799 (18 target genes) and microarray analysis for the identification of staphylococci at species-level, for the  
800 detection of resistance genes and for the detection of the Panton-Valentine leukocidin (PVL) with a time  
801 to result of about 5 hours. The overall accuracy for the detection of staphylococci at the species level from  
802 mono-bacterial or poly-bacterial culture was 91.7% when compared to conventional methods (230). The  
803 StaphPlex exhibited 100% of sensitivity and 95.5% to 100.0% of specificity for the detection of the *mecA*  
804 gene. Similar results were obtained for the detection of the PVL gene (230).

805 The FilmArray (Idaho Technology, Salt Lake City, UT, USA) is another PCR-based system that can  
806 detect the *S. aureus mecA* gene as well as the *vanA* and *vanB* genes implicated in vancomycin resistance  
807 in *Enterococcus* species (16; 192). The FilmArray system exhibits a high sensitivity and specificity  
808 (100%) for the detection of the *mecA* gene from *S. aureus* positive culture with (16; 19; 196). Another  
809 study reported 96% of sensitivity and 98.9% of specificity for the detection of the *mecA* gene (6). For the  
810 detection of the *vanA* and *vanB* genes in *Enterococcus spp.*, Blasche *et al.* reported only 85% of  
811 specificity of the FilmArray system when compared to BC (19). In this study, the FilmArray detected a  
812 *van* gene in 2 *Enterococcus* strains that were found vancomycin sensitive by culture based methods. The  
813 second strain was actually an *Enterococcus casseliflavus* strain bearing the *vanC* gene. During the  
814 development of the system, more resistance markers have been successfully tested (19).

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

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

## 823 **Detection of resistance mechanisms of gram-negative bacteria**

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

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

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

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

### 851 **3. Resistance detection directly from whole blood**

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

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

867

### 868 **CONCLUSIONS AND FUTURE DIRECTIONS**

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

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

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

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



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

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

909 Regarding poly-microbial identification, the quantification provided by some nucleic-acids based  
910 methods, can be helpful at determining the significance of the respective organisms. Another research  
911 focus is the attempt to provide clinical scores that would predict the severity of bacteremia (78; 195). The  
912 time to positivity of blood BC has been proposed as a criterion for BSI severity (7; 113; 129). Similarly,  
913 quantification could provide some insight on the severity of the BSI. Thus, analysis of 250 whole-blood  
914 samples from 20 adult patients (13 survivors and 7 nonsurvivors) with culture-proven MRSA showed that  
915 the levels of *mecA* DNA was higher in the nonsurvivors (5.48 copies/ml) than in the survivors (4.58 log  
916 copies/ml P= 0.003, two-tailed Mann-Whitney U test). This suggested that the level of *mecA* DNA in  
917 blood could potentially be used to monitor MRSA bacteremia and evaluate responses to therapy (109).  
918 Similarly a positive correlation has been found between the level of *Streptococcus pneumoniae* DNA  
919 monitored by real-time PCR and the need of mechanical ventilation, the risk of septic shock and of death  
920 (32; 198). Another study performed with the SeptiFast on 94 patients reported that the median cycle  
921 threshold (Ct) value was 16.9 for patients with severe septic choc and 20.9 for patients with non-severe

922 sepsis and that Ct value <17.5 correlated with more positive blood culture and longer hospital stays (80;  
923 266).

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

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

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

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

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952

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1740 **TABLES AND FIGURES**

1741

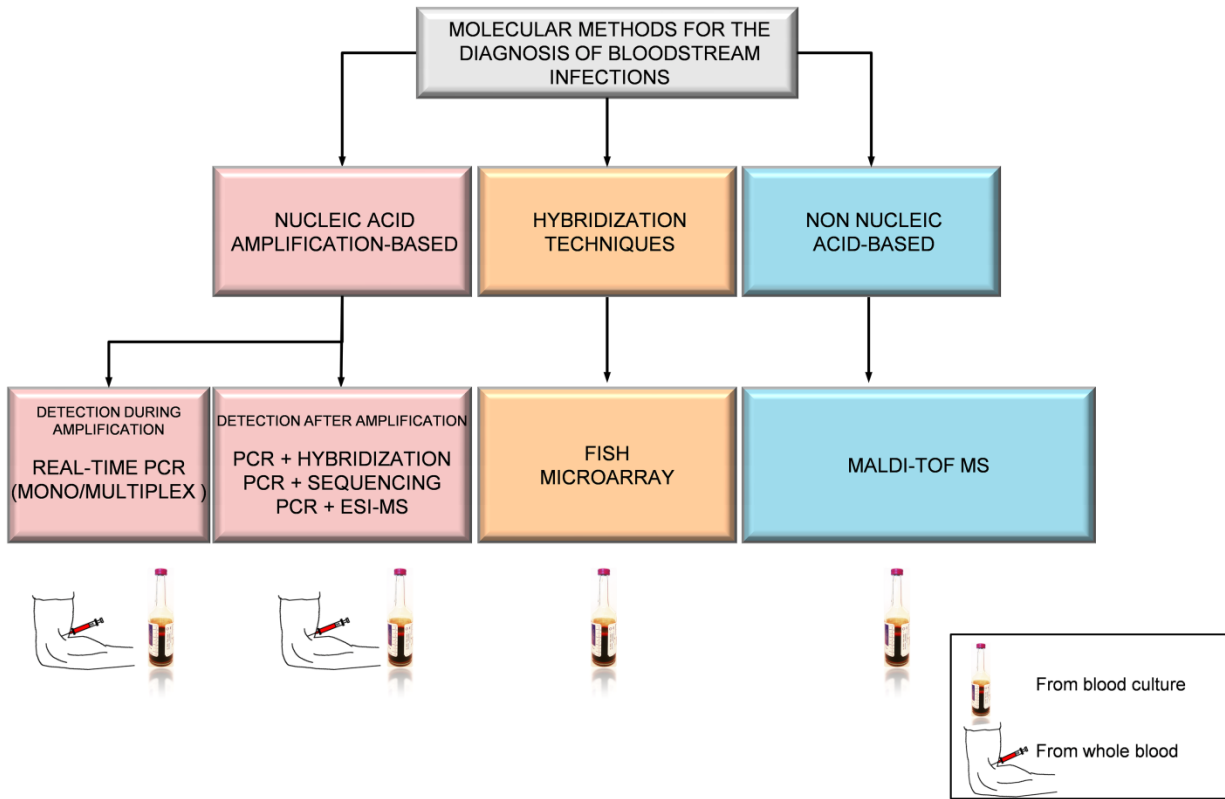
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1743 **Table 1: Commercially available molecular systems for the microbial identification of pathogen**  
 1744 **during BSI.** Adapted from (174; 175)

1745

| SYSTEM (MANUFACTURER)  | METHODS                               | TIME TO RESULT                   | MICROORGANISM COVERAGE   | RESISTANCE AND VIRULENCE MARKERS  | SENSITIVITY, SPECIFICITY AND CORRELATION WITH CONVENTIONAL METHODS | REFERENCES  |
|--|---------------------------------------|----------------------------------|--|---|--|---|
| <b>IDENTIFICATION FROM POSITIVE BLOOD-CULTURE</b>                                      |                                       |                                  |  |   |  |   |
| PNA FISH and QuickFISH (AdvanDx, Wolburn, MA)  | FISH                                  | <1-3 hours                       | 4 gram-negative<br>4 gram-negative<br>5 fungi  | none  | 97-100%<br>90-100%<br>96-99%                                       | (31; 55; 56; 85; 103; 106; 167; 220; 225)   |
| AccuProbe (Gen-Probe, San Diego, CA, USA)  | FISH                                  | <1 hour                          | <i>S. aureus</i><br><i>Enterococcus</i> spp.<br><i>S. pneumoniae</i><br><i>Streptococcus</i> group A<br><i>Streptococcus</i> group B | none  | 80.8-100%<br>98.7-100%<br>nr                                       | (9; 151)  |
| Verigene (Nanosphere, Northbrook, IL, USA)   | Microarray                            | 2.5 hours                        | 12 gram-negative<br>9 gram-negative  | <i>mecA</i> , <i>vanA/B</i> ,<br><i>bla<sub>KPC</sub></i> , <i>bla<sub>NDM</sub></i> , CTX-M, VIM, IMP, OXA12 | 81-100%<br>98-100%<br>nr   | (5; 14; 17; 26; 60; 155; 165; 226; 227; 234)  |
| Prove-it Sepsis (Mobidiag, Finland)  | Microarray                            | 3.5 hours                        | 60 bacteria<br>13 fungi  | <i>mecA</i>   | 95 %<br>99 %<br>nr   | (233)   |
| FilmArray (Idaho Technology, Salt Lake City, UT, USA)                                  | Multiplex PCR                         | 1 hour                           | 8 gram-negative<br>11 gram-negative<br>5 fungi   | <i>mecA</i> , <i>vanA/B</i> ,<br><i>bla<sub>KPC</sub></i>   | 97-95%<br>91-98%<br>nr   | (6; 19; 181; 196)   |
| Xpert MRSA/SA BC (Cepheid, Sunnyvale, CA, USA)   | Real-time PCR                         | 1 hour                           | <i>S. aureus</i>   | <i>mecA</i>   | 100%<br>99-100%<br>nr  | (45; 54; 140; 208; 221)   |
| StaphSR assay (BD GeneOhm, San Diego, CA, USA)   | Multiplex PCR                         | 1-2 hours                        | <i>S. aureus</i>   | <i>mecA</i>   | 96-100%<br>95-98%<br>nr  | (97; 130)   |
| StaphPlex (Genaco Biomedical Products, Huntsville, AL, USA)                            | Multiplex PCR + Microarray            | 5 hours                          | <i>S. aureus</i>   | <i>mecA</i> (+ PVL)   | 100%<br>95-100%<br>92%   | (230)   |
| MALDI-TOF MS Bruker Daltonics (Bremen, Germany) or bioMérieux (Marcy l'Etoile, France) | Mass-spectrometry                     | <1 hour                          | <1000 <sup>a</sup>   | not in routine  | -<br>-<br>76-99%   | (42; 52; 156; 158; 193; 194; 224)   |
| <b>IDENTIFICATION FROM WHOLE BLOOD</b>   |                                       |                                  |  |   |  |   |
| SepsiTest (Molzylm, Bremen, Germany)   | Broad range PCR + sequencing          | 6 hours (1-10 <sup>b</sup> ml)   | >345 bacteria and fungi  | none  | 21-87%<br>85-96%<br>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<br>8 gram-negative<br>5 fungi  | <i>mecA</i> <sup>b</sup>  | 43-95%<br>60-100%<br>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)<br>6 fungi  | <i>mecA</i> , <i>vanA/B</i>   | 37-65%<br>77-92%<br>73%  | (30; 152)   |
| VYOO (SIRS-Lab, Jena, Germany)   | Multiplex PCR + electrophoresis       | 8 hours (5ml)                    | 14 Gram positive<br>18 gram-negative<br>7 fungi  | none  | nr<br>nr<br>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 <sup>c</sup> ml) | up to 800 (Gram positive, Gram negative, fungi)  | <i>mecA</i> , <i>bla<sub>KPC</sub></i> ,<br><i>vanA/B</i>   | 50-91% <sup>d</sup><br>98-99%<br>79-97%                            | (13; 166)   |

1746 <sup>a</sup> Dependant on the mass-spectrum database  
1747 <sup>b</sup> With an additional kit  
1748 <sup>c</sup> For the latest version  
1749 <sup>d</sup> depending on the volume  
1750 nr=non reported  
1751



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

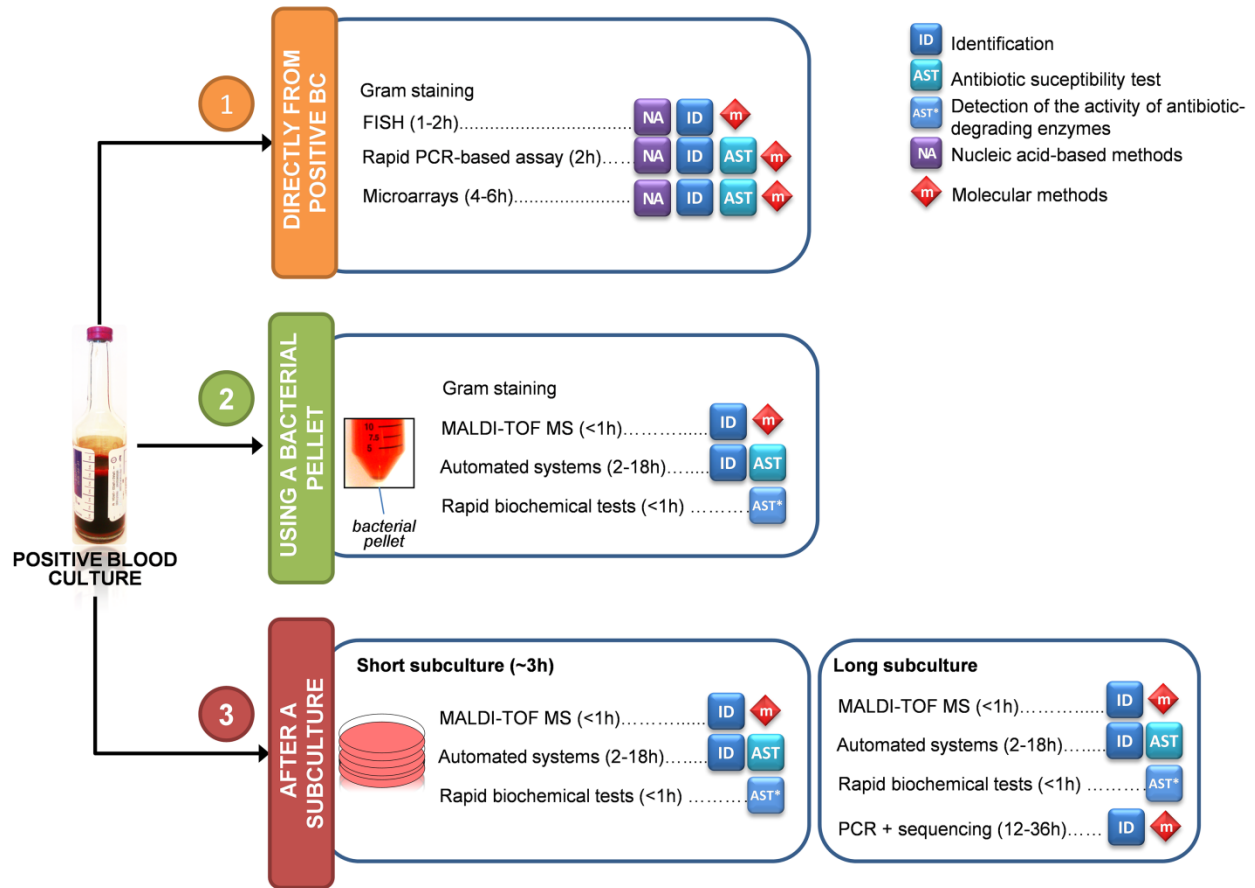
1756 positive blood cultures or used directly on blood whereas non nucleic acid amplification-based techniques

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



1761

1762

1763 **Figure 2: Conventional and molecular methods for the identification of microorganisms from**

1764 **positive blood culture.** When a blood culture is detected as positive several strategies are available to

1765 identify the incriminated microorganism after the initial Gram staining: (1) Identification directly from

1766 the positive blood culture using nucleic acid-based methods, (2) Identification after microbe's enrichment,

1767 namely a purified bacterial pellet suitable for MALDI-TOF MS analysis and some automated

1768 identification/AST approaches such as Vitek2 and Phoenix and rapid biochemical tests HMRZ and

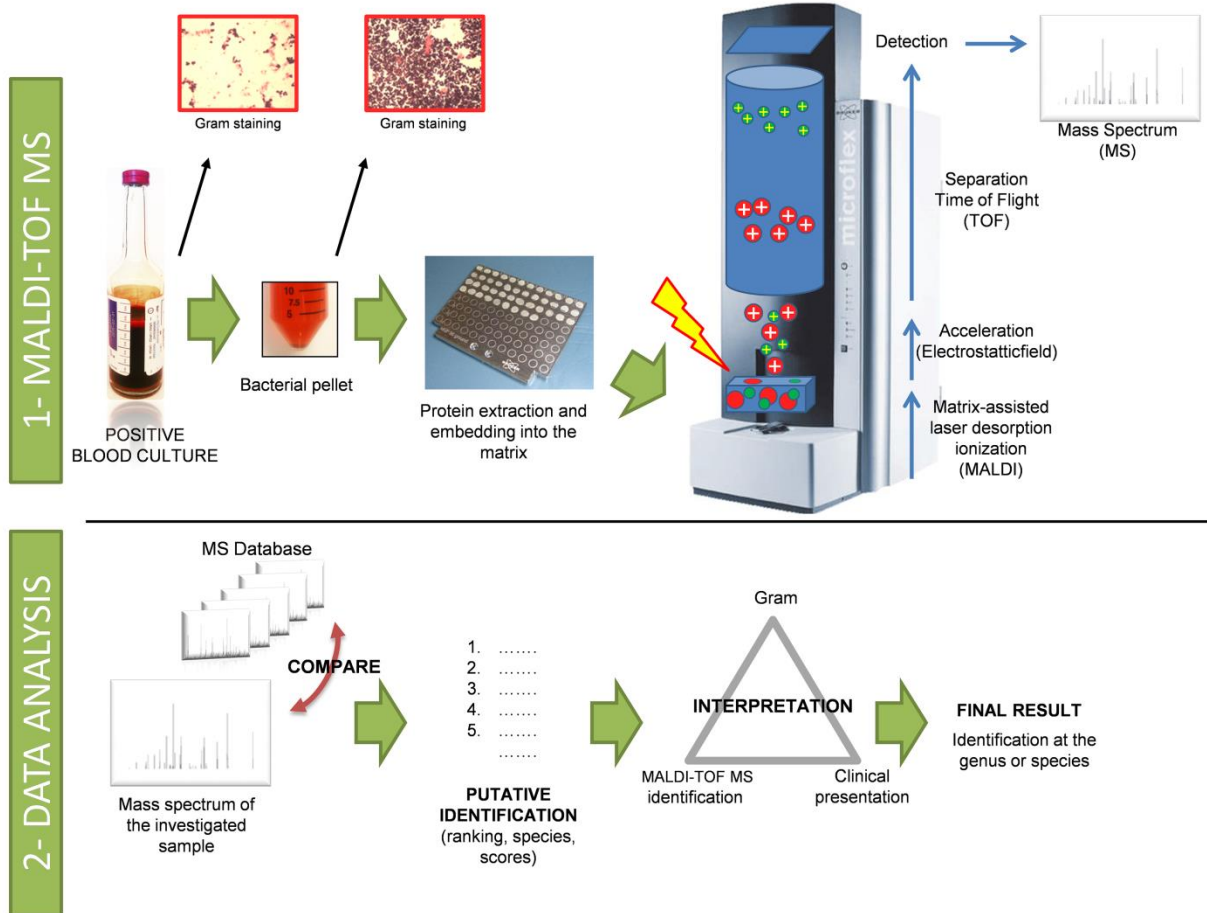
1769 ESBLNP (3) Identification after a subculture; to date the automated system Vitek2 has been validated on

1770 short subculture as well as the rapid biochemical tests HMRZ and CARNP; long subculture are suitable

1771 for any type of analysis including phenotypic characterization, automated systems such as Vitek2,

1772 Phoenix and MicroScan WalkAway as well as PCR followed by sequencing. Adapted from (174).

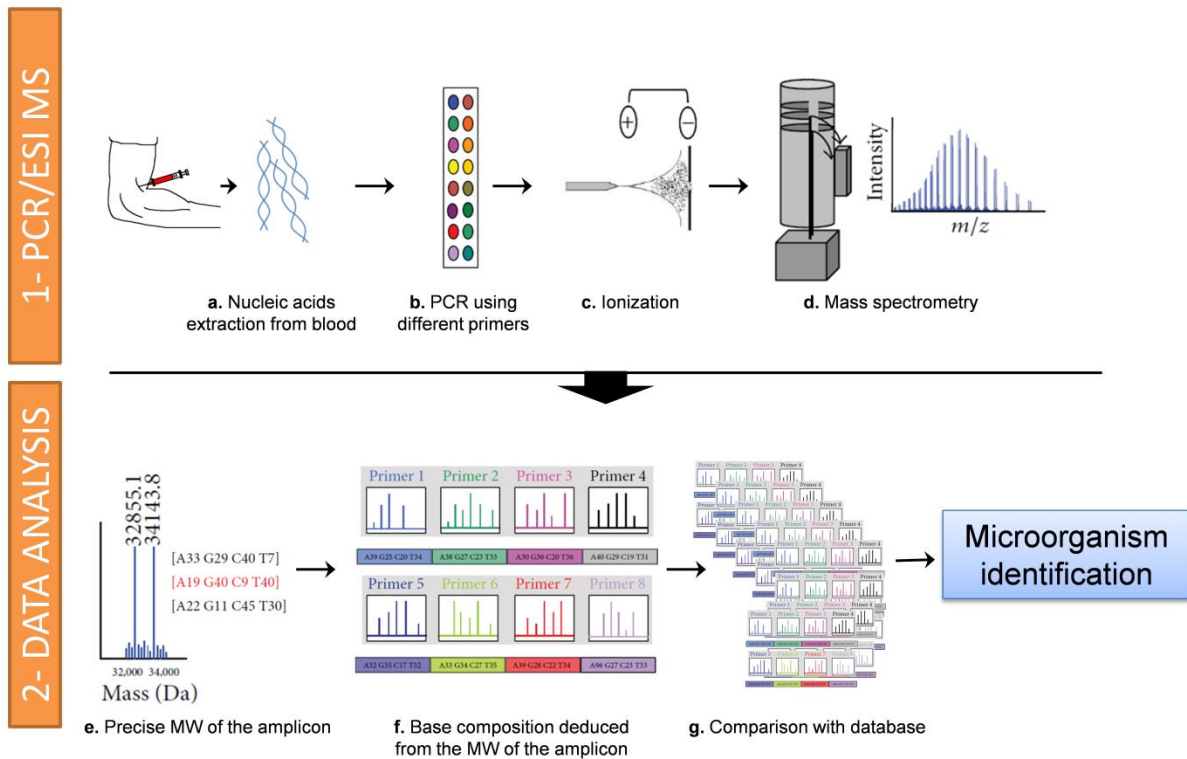
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1774

1775 **Figure 3: Microbial identification from positive blood culture using MALDI-TOF MS.** The sample  
 1776 that is deposited on the MALDI-TOF multi-well plate can be a bacterial pellet obtained by centrifugation  
 1777 and erythrocytes lysis. Alternatively, the sample can be bacteria obtained after a subculture. Proteins are  
 1778 then extracted and embedded in a matrix directly on the multi-well conductive metal plate and submitted  
 1779 to the MALDI-TOF MS, which separate the proteins according to their MW and their charges. This  
 1780 generates a mass spectrum (MS) which, in a second stage is compared to a database of spectra. This  
 1781 analysis provides the identification of the microorganism with a confidence score, which allows  
 1782 acceptance at the species or at the genus level. The identification is interpreted according to the Gram  
 1783 staining and to the clinical presentation. Adapted from (52).

1784



1785

1786

1787 **Figure 4: Schematic workflow of PCR/ESI-MS.** (a) Nucleic-acids are extracted from the sample,  
 1788 directly from whole blood, and (b) amplified by multiple PCR using multiple pairs of primers; each  
 1789 colour represents a different primer. After amplification, the molecular mass of the amplicon(s) is  
 1790 precisely determined using ESI-MS (c, d and e), from which (f) the base composition of the amplicon(s)  
 1791 is deduced. Finally, (g) informations - base composition - obtained from one or more amplicon(s) are  
 1792 compared with a database, which provides the identification with a confidence score. Adapted from (123).

1793