UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA:

CIÊNCIAS MÉDICAS

RAPID BACTERIA IDENTIFICATION AND SUSCEPTIBILITY PROFILE IN BLOOD CULTURE FROM PATIENTS ATTENDING AT A TERTIARY CARE HOSPITAL IN SOUTH OF BRAZIL.

OTIMIZAÇÃO DA ROTINA DE HEMOCULTURAS POSITIVAS NO LABORATÓRIO DE MICROBIOLOGIA: INTEGRAÇÃO DA IDENTIFICAÇÃO BACTERIANA E TESTE DE SUSCETIBILIDADE AO ANTIMICROBIANOS, UMA ABORDAGEM SIMPLES E RÁPIDA PARA REDUZIR O TEMPO DE LIBERAÇÃO.

DARIANE CASTRO PEREIRA

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“Do not wait: the time will never be 'just right'.
Start where you stand, and work whatever tools you may have at your command and better tools will be found as you go along.”
— Napoleon Hill

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ABSTRACT

Background. Bloodstream infection is a critical disease with high mortality rate. Adequate treatment required rapid microbiological identification and timely administration of appropriate antibiotics. Rapid diagnostic tests that accurately identify infection-causing pathogens and the effective antimicrobials against these pathogens can increase the likelihood that patients are treated appropriately. Rapid diagnostic tests can also be used to help clinicians discontinue unnecessary antibiotics or de-escalate broad-spectrum antimicrobial therapy to a narrower-spectrum option. Current technologies employed in blood culture routine diagnostics are precise and sensitive but rather slow, most depending on bacterial growth. **Objective.** In this study, we evaluated a rapid bacterial identification (rID) and a rapid antimicrobial susceptibility testing by disk diffusion (rAST) from positive blood culture to overcome the limitations of the conventional methods. We investigated a rapid workflow to reduce the turnaround time in bloodstream infections diagnostics in a routine microbiology laboratory. **Methods**. The study included hemocultures flagged as positive by BACT/ALERT® (Biomérieux, Marcy l'Etoile, France) between 7-12:00 a.m. and inoculated on Chocolate agar at our clinical microbiology laboratory. At 16.00 p.m., after 4-6h incubation at 35-38°C (5% CO_2), identification by MALDI-TOF MS (VITEK MS® system,Biomérieux, Marcy-l'Étoile, France) and AST (disk diffusion method) were performed. Results were compared to identification (sID) and AST (sAST) results after 24h incubation. An identification score value of > 96% was considered as a correct species identification. For AST categorical agreement (CA), very major errors (VME, false-susceptible result of rapid AST), major errors (ME, false-resistant result of rapid AST), minor errors (mE, false categorization involving intermediate result) were investigated. **Results.** We identified a total of 526 bacterial isolated from blood cultures obtained from patients attended at a tertiary

hospital in the south of Brazil, 246 Gram-negative (GN) and 279 Gram-positive (GP) aerobes. The overall concordance between rID and sID was 88.6% and was highest for GN (95.5%). *K. pneumoniae* and *E. coli* presented 96.0% and 98.5% rate of concordance, respectively. Total of 2196 and 1476 antimicrobial agents' comparisons were obtained for GN and GP, respectively. Evaluating rAST, the CA, VME, ME and mE were 97.7, 0.7, 0.5 and 1.1% for GN and 98.0, 0.5, 0.7 and 0.8% for GP, respectively. Meropenem CA, VME and ME were 98.3, 0.5 and 0.5%, respectively; no mE was observed. Oxacillin CA, ME and mE were 97.4, 1.6 and 0.6%, respectively; no VME was observed. Sensitivity and specificity of rAST method were calculated for each antimicrobial agents. Meropenem presented 99.2 and 98.1% and Oxacillin presented 96.9 and 97.9% of sensibility and specificity, respectively. Overall Kappa scores of the comparisons results demonstrated the high agreement between rAST and sAST. **Conclusions.** The rapid methods for bacterial identification and for rapid AST results proposed in this study were distinguished from standards ones by feasible modifications and the accuracy of rAST were comparable with the standard method. Identification and AST of aerobic bacteria from positive blood cultures after a shortened incubation on solid blood agar is a fast and reliable method that may improve management of bloodstream infections, allowing us to save up to 24 h to identifying bacteria and supply useful information to adapt antibiotic therapy when necessary.

Keywords: blood culture; rapid identification; MALDI-TOF; rapid antimicrobial resistance detection; disk diffusion

RESUMO

Introdução. A infecção da corrente sanguínea é uma doença crítica com alta taxa de mortalidade. O tratamento adequado exige identificação microbiológica rápida e administração oportuna de antibióticos apropriados. Testes diagnósticos rápidos que identifiquem com precisão os patógenos causadores de infecções e os antimicrobianos eficazes contra esses patógenos podem aumentar a probabilidade dos pacientes serem tratados adequadamente. As tecnologias atuais empregadas nos diagnósticos de rotina da hemocultura são precisas e sensíveis, mas bastante lentas, e dependente do crescimento bacteriano em cultura em meio sólido. **Objetivo**. Neste estudo, nós avaliamos a identificação rápida bacteriana (rID) e um teste rápido de susceptibilidade antimicrobiana por disco-difusão (rAST) de hemocultura positiva para otimizar a rotina de hemoculturas positivas, superando as limitações dos métodos convencionais. Nós investigamos um fluxograma rápido para reduzir o tempo de liberação dos resultados no diagnósticos de infecções da corrente sanguínea em um laboratório de microbiologia clínica. **Métodos** Hemoculturas positivas pelo BACT / ALERT® (Biomérieux, Marcy l'Etoile, França) entre as 7 - 12:00h eram processadas e inoculadas em ágar Chocolate. Após 4-6h de incubação a 35-38 ° C (5% CO2), identificação por MALDI-TOF MS (sistema VITEK MS®, Biomérieux, Marcy-l'Étoile, França) e AST (método de difusão em disco) foram realizados. Os resultados foram comparados com os resultados de identificação (sID) e AST (sAST) a partir de culturas de 24h. Para a concordância categórica AST (CA), erros muito importantes (VME, resultado falso-suscetível da AST rápida), erros maiores (ME, resultado falso-resistente da AST rápida), erros menores (mE, falsa categorização envolvendo resultado intermediário) foram investigados. **Resultados**. Identificamos um total de 526 bactérias isoladas de hemoculturas obtidas de pacientes atendidos em um hospital terciário no sul do Brasil, 246 aeróbios Gram-

negativos (GN) e 279 Gram-positivos (GP). A concordância global entre rID e sID foi de 88,6% e foi mais alta para GN (95,5%). *K. pneumoniae* e *E. coli* apresentaram taxa de concordância de 96,0% e 98,5%, respectivamente. Total de 2196 e 1476 comparações de agentes antimicrobianos foram obtidas para GN e GP, respectivamente. Avaliando rAST, o CA, VME, ME e mE foram 97,7, 0,7, 0,5 e 1,1% para GN e 98,0, 0,5, 0,7 e 0,8% para GP, respectivamente. Para meropenem CA, VME e ME foram 98,3, 0,5 e 0,5%, respectivamente; nenhum mE foi observado. Para oxaciina CA, ME e mE foram 97,4, 1,6 e 0,6%, respectivamente; nenhum VME foi observado. A sensibilidade e especificidade do método rAST foram calculadas para cada agente antimicrobiano. Meropenem apresentou 99,2 e 98,1% e a Oxacilina apresentou 96,9 e 97,9% de sensibilidade e especificidade, respectivamente. Os escores Kappa gerais dos resultados das comparações demonstraram a alta concordância entre rAST e sAST. **Conclusão.** Os métodos rápidos para identificação bacteriana e para teste de sensibilidade aos antimicrobianos propostos neste estudo diferenciam-se dos padrões por modificações no tempo de cultura e a precisão do rAST foi comparável com o método padrão. Identificação e AST de bactérias aeróbias de hemoculturas positivas após uma incubação rápida em meio sólido é um método rápido e confiável que pode otimizar o diagnóstico de infecções de corrente sanguínea, permitindo-nos reduzir em até 24 h a liberação da identificação bacteriana e o perfil de sensibilidade, assim fornecer informações de forma mais rápida e precisas a equipe assistencial.

Palavras-chave: cultura do sangue; identificação rápida; MALDI-TOF; detecção rápida de resistência antimicrobiana; difusão de disco

LIST OF ABREVIATIONS

AST	Antimicrobial susceptibility test
BSI	Bloodstream infection
CA	Categorical agreement
CLSI	Clinical Laboratory Standards Institute
CNS	coagulasse negative *Staphylococcus*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MS	Mass spectrometry
ME	Major Error
mE	Minor Error
MDR	Multidrug resistance bacteria
MIC	Minimum inhibitory concentration
rID	Rapid bacterial identification
rAST	Rapid antimicrobial susceptibility testing
SENTRY	Antimicrobial Surveillance Program
sAST	Standard antimicrobial susceptibility testing
sID	Standard bacterial identification
SIRS	Systemic Inflammatory Response Syndrome
TAT	Tourn Around Time
VME	Very Major Error

LIST OF FIGURE

SUMMARY

1. INTRODUCTION

Rapid acquisition of blood culture pellet, coupled with a rapid short-time subculture allowing bacterial identification and phenotypic determination of the antibiotic susceptibility, could significantly reduce the time to results, greatly benefitting patient care by reducing mortality and morbidity as well as overall costs for health care systems (1-3). Identification of microorganisms in a clinical microbiology laboratory provides definitive knowledge about the cause of infection and plays a critical role in patient management and choice of antimicrobial treatment. The conventional methods for identifying microorganisms are based on phenotypic methods, such as growth on selective and non-selective media, morphology of colonies, Gram-stain and typical biochemical reactions using automated or manual testing methods (4). Because these methods often rely on active metabolic processes of the involved microorganisms, growth and therefore long incubation periods are sometimes needed requiring a relatively long turn-around time and the results may be difficult to interpret occasionally, because of indistinct reactions or outdated databases.

Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) emerged as a rapid, accurate, cost-effective method and has been effectively used as a rapid method for identifying a wide array of microbial species (5). In MALDI-TOF MS analysis, abundant structural proteins such as ribosomal proteins are extracted from an intact bacterial colony. The ionizing laser vaporizes the abundant structural proteins of microorganisms, and unique mass spectra are generated, having mass-to-charge ratio (m/z) peaks with varying intensities. The mass spectra of test isolates are sequentially compared with those in a reference database for identification (6). In comparison with conventional methods for identification of clinical samples by and despite its high-end technology, a MALDI-TOF MS device is simple to

use. MALDI-TOF MS can provide advantages for a universal procedure of microbial identification. Only a small amount of an organism, typically a fraction of a single colony from primary culture plates, is required for analysis. Comparatively, a larger inoculum and subculture is often required for conventional biochemical methods or other automated systems. Furthermore, once the instrument is loaded, identifications can typically be performed in less than one minute, compared with hours to days for conventional methods (6, 7).

MALDI-TOF MS is an efficient and reliable method for identification of bacteria and the clinical microbiology literature is replete with publications demonstrating the excellent performance of MALDI-TOF MS for routine organism identification (7-10). According to literature the identification rates of genus are extremely high (97-99%) and varies from 85% to 97% at the level of species (10). A study by Cherkaoui et al. (9) compared two commercially available MALDITOF MS systems (Bruker Daltonics and Shimadzu) for phenotypic bacterial identification of 720 samples representing 33 different genera. In their setting, correct identification at the species level by MALDI-TOF MS was obtained in 93.6% of cases for the Bruker and 88.3% for the Shimadzu. Seng et al. (7) showed that of 1660 bacterial isolates representing 109 different species, 84.1% were correctly identified by MALDI-TOF MS at the species level and 11.3% at the genus level only.

The main use of MALDI-TOF for microorganisms' identification is growing the bacteria in culture medium which takes 18–24 h. This is the standard method, manufacturer recommendation, diffused in most microbiology centers. Considering that rapid identification of microorganisms in blood culture is crucial for the managing septicemia due to its high disease severity, and direct identification from blood culture bottles can shorten the turnaround time, various studies have used MALDI-TOF MS for identification of microorganisms directly from positive blood cultures and

demonstrated identification rates of 59-90% (10). Various research protocols for microorganism identification directly from blood culture have been used with a degree of success in microbial identification to genus or species level using MALDI-TOF, including lysis filtration (11, 12), Sepsityper (13) or in-house saponin-based bacterial extraction (14, 15). The methods described include differential centrifugation, washing, gel-based separation and protein extraction steps to isolate bacteria and remove substance that can interfere with MALDI-TOF analysis. However, direct identification from positive blood cultures must be simple, fast, inexpensive, in order to be easily adopted by clinical microbiology laboratories. Henceforth, a rapid short incubation on solid media of blood culture pellet is a simple protocol and feasible for routine practice with appropriate accuracy and performance comparable to that direct vials identification methods (16-21).

Additionally, rapid and accurate antimicrobial susceptibility test (AST) is paramount to the management of patients with serious infections. The ability to report identification and susceptibility results from positive blood cultures shortly after they signal positive for growth is of great value in reducing time to appropriate therapy (6, 22). Otherwise, the current culture-based AST tools rely on time-consuming culturing techniques, followed by disk-diffusion and broth dilution susceptibility testing. In many clinical microbiology laboratories, agar disk diffusion is routinely used, while automated AST instruments are limited in the number of antibiotics, concentrations tested and lack the capability of analyzing polimicrobial samples or heterogeneous response of bacterial populations to the antibiotics (23). Considering these, there is an onus on clinical microbiology laboratories to provide a more rapid robust susceptibility testing so as to rapid bacterial identification from blood cultures (24, 25)

Rapid microbiology tests may contribute to the early treatment of patients by using an appropriate antimicrobial therapy, thereby improving patient outcomes reducing the potential for microorganisms to develop antimicrobial resistance, and lowering

mortality among patients with sepsis (6). Moreover, most of these previous studies focused on direct bacterial identification, with little application of a direct culture-independent AST method. Therefore, we developed a simple and feasible protocol integrating rapid bacterial identification from positive blood cultures by using MALDI-TOF MS, following a rapid AST test by modification of the standardized disc diffusion method. This protocol results in definitive bacterial identification and AST results 24-48 hours earlier than is possible with the standard protocol.

2. REVIEW OF LITERATURE

2.1 Search strategy and Concept map

An online search of the PubMed-Medline databases was performed up to June 2018 to identify studies using protocols for rapid bacteria identification from blood culture by MALDI-TOF and also rapid antimicrobial susceptibility testing. The terms and descriptors used in the searching process were selected based on the keywords available in previous studies via Mesh terms. The descriptors of Mesh were:

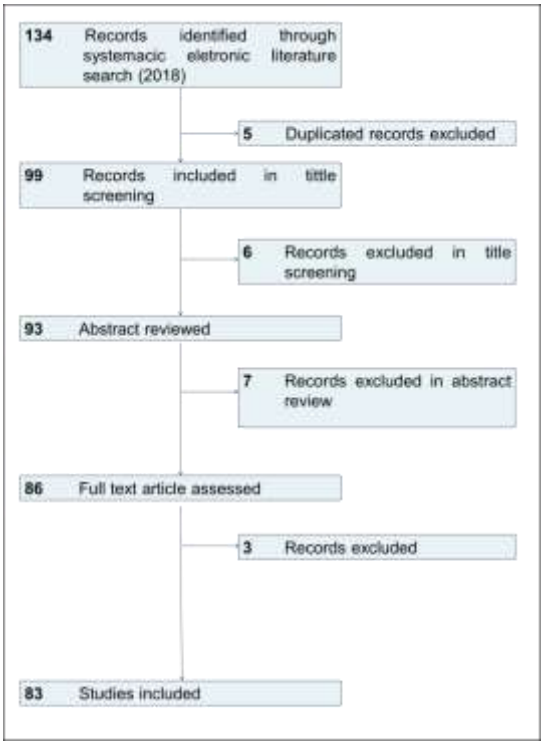

Fig.1. Flowchart of literature review procedure. The literature was reviewed in a five stage process, followed by four screens of increasing detail, reviewing the article title, abstract and full text. A total of 83 articles were included.

“bacteremia/diagnosis”, “Matrix-Assisted Laser desorption-ionization” OR “MALDI-TOF”, “blood culture”, “Disk diffusion antimicrobial tests” OR “Microbial Sensitivity Tests”, “rapid antimicrobial resistance detection”. The results are showed in Figure 1.

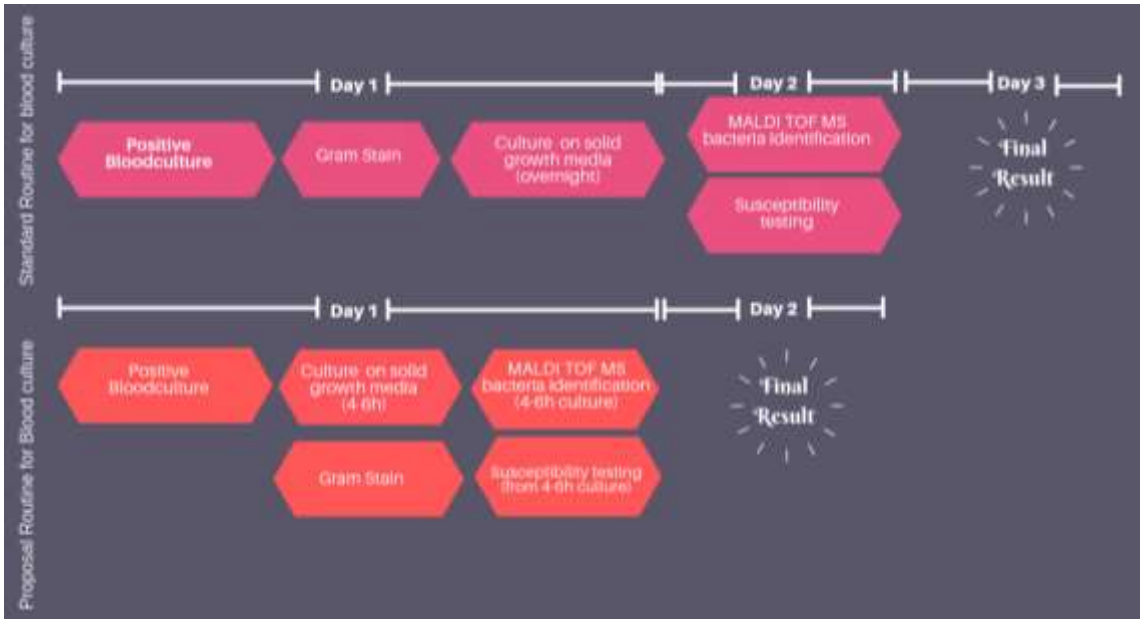

Fig. 2. Concept map.

2.2 General aspects of Bloodstream infections and sepsis

2.2.1 Definitions

Bloodstream infection is a condition where bacteria or fungi are isolated from blood cultures from a patient with clinical signs and symptoms of systemic infection, and where contamination has been ruled out. Bloodstream infection includes the terms bacteremia and fungemia.

Until 1990, sepsis was defined as the presence of pathogenic organisms or their toxins in the blood or tissues (26). During the 1980s, however, an increasing knowledge in sepsis pathophysiology gave the understanding of sepsis as a state driven by a dysregulated host response (27). Therefore, in 1991, a consensus conference (28) proposed a new definition of sepsis: The sepsis-1 definition 1991 The conference

introduced the term Systemic Inflammatory Response Syndrome (SIRS) and defined sepsis as a clinical syndrome with both infection (detected or suspected) and SIRS. If two or more of the following criteria are fulfilled, a patient has SIRS: (i) Temperature >38.0ºC or lower than 36. 0ºC; (ii) Heart rate >90 per minute; (iii) Hyperventilation evidenced by respiratory rate >20 per minute or pCO_2 $12x10^{-9}$ per Liter or 10% immature neutrophils.

Because of increasing discontentment with the sepsis-1 definition, an extended consensus conference was held in 2001 (29). The 2001-definition of sepsis (Sepsis-2 definition) was detected or suspected infection plus other criteria from the following categories of variables:

- General variables: (i) Temperature > 38.3^{o} C or 90 beats/min (ii) Tachypnoe>20 breaths /min or $PaCO_2$ 20 ml/kg over 24 h (iii) Hyperglycemia in the absence of diabetes. Plasma glucose > 7.7 mmol/L.
- Inflammatory variables: (i) Leukocytosis (>12 x 10^9 /L) or leucopenia (< $4x10^9$ /L) or >10% immature neutrophils (ii) Plasma C reactive protein (CRP) >2 SD above the normal value (iii) Plasma procalcitonin > 2 SD above the normal value.
- Hemodynamic variables: (i) Arterial hypotension (systolic blood pressure < 40. Partial oxygen pressure in arterial blood (kPa) divided by the fraction of oxygen in inspired air (0.2 in atmospheric air).
- Organ dysfunction variables: (i) Arterial hypoxemia. PaO_2/FiO_2 < 40. Partial oxygen pressure in arterial blood (kPa) divided by the fraction of oxygen in inspired air (0.2 in atmospheric air). (ii) Use of mechanical ventilation for acute respiratory failure; (iii) Acute oliguria; (iv) Creatinine increase > 45 µmol/L; (v) Coagulation abnormalities (international normalized ratio (INR) >

1.5 or activated partial thromboplastin time (APTT) >60 sec); (vii) Thrombocytopenia (platelet count 70 Pmol/L).

- Tissue perfusion variables: (i) Decreased capillary refill (> 3 seconds); (ii) Mottling; (iii) Hyperlactatemia (> 3 mmol/L).

Severe sepsis represented a state of suspected infection plus some degree of organ dysfunction (29). In the Sepsis-3 definition, the term severe sepsis is no longer in use (30). Septic shock according to the Sepsis-3 definition, patients with septic shock can be clinically identified by a vasopressor requirement to maintain a mean arterial pressure of 65 mmHg or greater and serum lactate level greater than 2 mmol/L in the absence of hypovolemia.

2.2.2 Clinical impact

Bloodstream infection (BSI) is still among the ten most common causes of deaths in developed countries (31). Incidence rates of BSI between 80 and 257 per 100,000 person-years have been reported, with higher rates in the more recent years (32, 33), and the case fatality rate constitutes 10-40% (34, 35). The severity of BSI is not only caused by pathogenic microbes, but is just as much a result of uncontrolled host response that damages the host's own organs and tissues - sepsis. About 25% of BSI patients develop sepsis, and 10% (32) to 16% deteriorate to septic shock (34).

Invasive bacterial and fungal infections if not treated effectively, they are life-threatening diseases. In spite of great treatment efforts during the last decades, infections are still among the most common causes of death worldwide. In the last decades, a rising occurrence of microorganisms resistant to antimicrobial agents is of particular concern (36). Patients infected with resistant microbes often receive appropriate antimicrobial therapy either too late or not at all. Consequently, more deaths are caused by infections that could formerly be treated successfully.

In developed countries, the incidence of severe sepsis has been estimated to 50 – 100 per 100,000 person-years (37). The incidence of sepsis according to the 2001-definition is 3-4 times higher, as about 25% of sepsis patients develop organ dysfunction. The case fatality rates are 20% - 50% in sepsis, and 40% - 80% in septic shock (37). During the last decades, the incidence of sepsis has been increasing in all areas of the world where epidemiologic studies have been conducted (37). An increasing number of older people with a high burden of comorbidity and more advanced health care, including invasive procedures and immunosuppressive therapy, are factors that contribute to increased incidence of BSI and sepsis. The increased incidence gives rise to increased mortality. In the months following BSI or sepsis, survivors have a significantly worse prognosis than population controls (secondary infections, persistent organ dysfunctions, vascular events, deaths) (38). Essential to patient survival is the administration of effective antibiotics, as data demonstrate that each hour a patient is on inappropriate therapy, their chance of survival decreases by 7.6%. Empirical treatment with broad-spectrum antibiotics is started in patients suspected of bacteremia; however, diagnostics are necessary, as recent studies have observed that 25 to 33% of patients are inappropriately treated within the first 24 h due to lack of coverage from organism's resistant to broad-spectrum antibiotics. To improve patient care, rapid bacterial identification and susceptibility results for blood cultures are needed (39, 40).

2.3 Microorganisms and antimicrobial resistance

The knowledge of microbial BSI is essential to ensure the appropriate antimicrobial and the best prognosis of these events. The microbiological profile of BSI is influenced by the environment of acquisition. Generally, the most common agents in BSI are *Staphylococcus aureus*, coagulase-negative *Staphylococcus* (CNS) and

Enterobacteriales. However, an estimate of each agent varies according to the site of study. According to BRAZILIAN SCOPE, which BSI data was acquired in hospitalized patients in the five regions of Brazil, gram-negative represented 58.5% of BSI between 2007 and 2010, with gram positive 35.4% and fungal 6.1%. The most common pathogens were *S. aureus* (14.0%), SCN (12.6%), *Klebsiella* spp. (12.0%), and *Acinetobacter* spp. (11.4%). The distribution of 16,949 microorganisms isolates reported as etiological agents in patients hospitalized at the Brazilian ITU in the year 2016 showed the predominance of SCN (18.9%), followed by *Klebsiella pneumoniae* (18.2%) and *S. aureus* (14.1%) (29).

In the last decades, hospital infections caused by multiresistant microorganisms have caused a significant increase in patients' morbidity and mortality, as well as a significant impact on care costs. The successive addition of genetic elements coding for aminoglycoside and wide-spectrum β-lactam resistance associated with the rapid accumulation of chromosomal mutations that also confer resistance to fluoroquinolones make carbapenems the drug of choice for the treatment of infections caused by gram-negative bacilli. This class of antibiotics has a bactericidal action and has been used extensively because they are stable drugs to most β-lactamases, such as AmpCs and Spectrum-Extended β-lactamases (ESBL) (41). Reports of resistance to these antibiotics were sporadic until the early 1990s, but since the last decade resistance to carbapenems has become an alarming problem and is considered a public health issue in several countries. The increasing resistance to these antibiotics is critical mainly in the case of the family Enterobacteriaceae and in the genera *Pseudomonas* spp. and *Acinetobacter* spp., due to the therapeutic restrictions available to treat the infections caused by these microorganisms.

Currently, the main mechanism by which Gram negative bacilli acquire resistance to carbapenems is through the production of enzymes called carbapenemases (42).They

are the most broad-spectrum β-lactamases and to date, more than 100 different carbapenemases have already been described (43, 44). KPC, in particular, is widespread worldwide and has been associated with outbreaks of multidrug-resistant Gram negative infections in many countries, including the most prevalent and clinically important carbapenemase in Brazil (45-47).

Antimicrobial resistance is recognized as one of the principal threats to public health throughout the world: its impact is felt in all areas of health, and it affects the whole society. Although antimicrobial resistance is a natural phenomenon, it is exacerbated by the misuse of antibiotic, poor-quality medicines, weak laboratory capacity, inadequate surveillance and poor regulation or enforcement of regulations to assure access to high-quality antimicrobial medicines and their appropriate use.

2.4 Diagnosis of bloodstream infections

2.4.1 Blood culture

In patients with suspected blood stream infections, at least 40 ml of venous blood should be inoculated in blood culture vials and then incubated in an incubation device with a temperature of 37°C until reported positive. Vials not reported positive will be reported negative after five days of incubation. BacT/ALERT® 3D (BioMérieux Inc., France) and BACTEC® (BD Diagnostic Systems, USA) are the most commonly used continuously monitored automated blood culture systems in many diagnostic microbiology laboratories. The main differences between the two blood culture systems are the composition of the growth-medium and also their different ways of neutralizing antibiotics potentially present in the blood sample. BacTEC® vials have resin and BacT/Alert® have charcoal to capture antibiotics. Studies have shown that there is no significant difference between the systems in detection of bloodstream infections in patients receiving antibiotics at the time of blood culture, so both resin and charcoal works equally well (48).

The two systems have similar principles for detecting growth. The systems detect CO_2 produced when microorganisms metabolize the substrate in the culture medium. The method of detection CO_2 is different in the two systems. Both types have a sensor at the bottom of the blood culture vial. In the BacTEC® system the produced CO_2 reacts with a pigment in the sensor. This reaction regulates the amount of light absorbed by a fluorescent material. A photodetector then measures the increased fluorescence, which is proportional to the concentration of CO_2 in the vial. In the BacT/Alert-system the bottom of each culture bottle visibly change color when the pH changes due to the rise in CO_2 as it is produced by microorganisms and the instruments measure the color changes every ten minutes and analyze the changes. (Fig. 3). A light emitting diode (LED) projects light on the sensor and the increased level of reflected light is registered by the photodetector.

Figure 3. Liquid Emulsion Sensors (LES) at the bottom of each culture bottle visibly change color when the pH changes due to the rise in CO_2 as it is produced by microorganisms in BACT/ALERT® instruments.

2.4.2 Bacterial identification

Traditionally, when a blood culture vial is positive a Gram stain is performed followed by a subculture on appropriate solid agar medium and different biochemical tests are performed manually. Using these conventional microbiological techniques, the time from positive signal in the blood culture system to species identification is at least 24 hours.

A variety of different platforms have been introduced to rapidly identify bacteria in positive blood culture bottles. Molecular techniques have been applied to speed up the species identification from positive blood culture bottles, such as nucleic amplification, in situ hybridization, and/or microarray technology (49). However, these systems are limited by the number of pathogens they can detect and require costly consumables ranging in price from tens to hundreds of dollars per sample. The estimated cost of analyzing 150 positive blood cultures using nucleic acid-based technology is between $10,000 and $20,000, assuming the organism is able to be detected by the platform (16).

Recently, MALDI-TOF MS has been used for identification of bacteria and yeasts and few developments in microbiological diagnostics have had such a rapid impact on species level identification of microorganisms as this methodology. MALDI-TOF MS allows identification of most of the pathogenic bacteria grown on agar plates from isolated colonies within a few minutes, and has proven efficiency and reproducibility. This radically new, methodically simple approach profoundly reduces the cost of consumables and time spent on diagnostics (7-10). In the study performed by Ge,M.C (2017) the use of MALDI-TOF yielded a 2.29-fold reduction in cost in reagent costs annually compared to traditional methods (6). Annually cost savings of 51.7%, by adopting the MALDI-TOF technology was observed by Tran et al (2015) when they

compared in the study the cost of performing the bioMérieux Vitek MALDI-TOF MS with that of conventional microbiological methods (50).

Although the initial instrumentation price is high and maintenance expenses are significant, the cost of identifying an isolate can be very low. MALDI-TOF MS not only represents an innovative technology for the rapid and accurate identification of bacterial and fungal isolates, it also provides a significant cost savings for the laboratory. Thus, MALDI-TOF MS is rapidly replacing conventional biochemical and phenotypic analytical methods for microbial identification in clinical microbiology laboratories around the world (51).

2.5 Mass spectrometry technology – MALDI -TOF

2.5.1 Principle of MALDI-TOF

Mass spectrometry is an analytical technique in which chemical compounds are ionized into charged molecules and ratio of their mass to charge (m/z) is measured. Though MS was discovered in the early 1900s, its scope was limited to the chemical sciences. However, the development of electron spray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) in 1980s increased the applicability of MS to large biological molecules like proteins. In MALDI peptides are converted into ions by either addition or loss of one or more than one protons, it is based on “soft ionization” method where ion formation does not lead to a significant loss of sample integrity (52).

MALDI-TOF MS instruments have 3 components: a specimen ionization chamber, a time-of-flight mass analyzer, and a particle detector (Fig.4). Sample preparation is simple, and involves transferring a portion of an isolated colony onto a target slide. The deposited colony is then covered with a chemical matrix and the target slide is loaded into the instrument. The sample-matrix mixture is pulsed by a laser, converting the sample into an ionic gas composed of small proteins and peptides and other molecules. In the ionization chamber, positively charged molecules are accelerated

through an electric field to velocities that depend on their mass-to charge (m/z) ratios. The particles then leave the electric field and enter the time-of-flight mass analyzer. The time it takes a particle to traverse the mass analyzer (“flight time”) depends on the velocity developed in the ionization chamber, and hence, on the m/z ratio. Flight times of individual particles are measured by a particle detector at the end of the mass analyzer, and are converted into m/z values that are plotted on a mass spectrogram. Identification of microbes by MALDI-TOF MS is done by either comparing the spectrogram of unknown organism with the spectrogram contained in the database library. For species level identification of microbes, a typical mass range m/z of 2–20 kDa is used, which represents mainly ribosomal proteins along with a few housekeeping proteins (8).

Principal advantages of MALDI-TOF MS technology include ease of use, potential to automate, rapid turnaround time, and low reagent costs. The simplicity of setup and the ability to run large numbers of isolates per batch readily lend this technique to high-throughput workflow and potential automation. Once the instrument is loaded, identifications can typically be performed in <1 minute, compared with hours to days for conventional methods. This improvement in turnaround time may carry substantial clinical benefit. Although purchase of a MALDI-TOF MS instrument involves a significant capital commitment with recurrent annual service contract fees, the reagents and disposables required consist primarily of target plates, microliter quantities of inexpensive organic compounds, inoculation loops, and pipette tips with estimated costs of as little as US$ 0.10 – $ 0.40 per identification when optimized. In some cases, this operational cost amounts to one-tenth that of conventional identification with automated biochemical testing platforms.

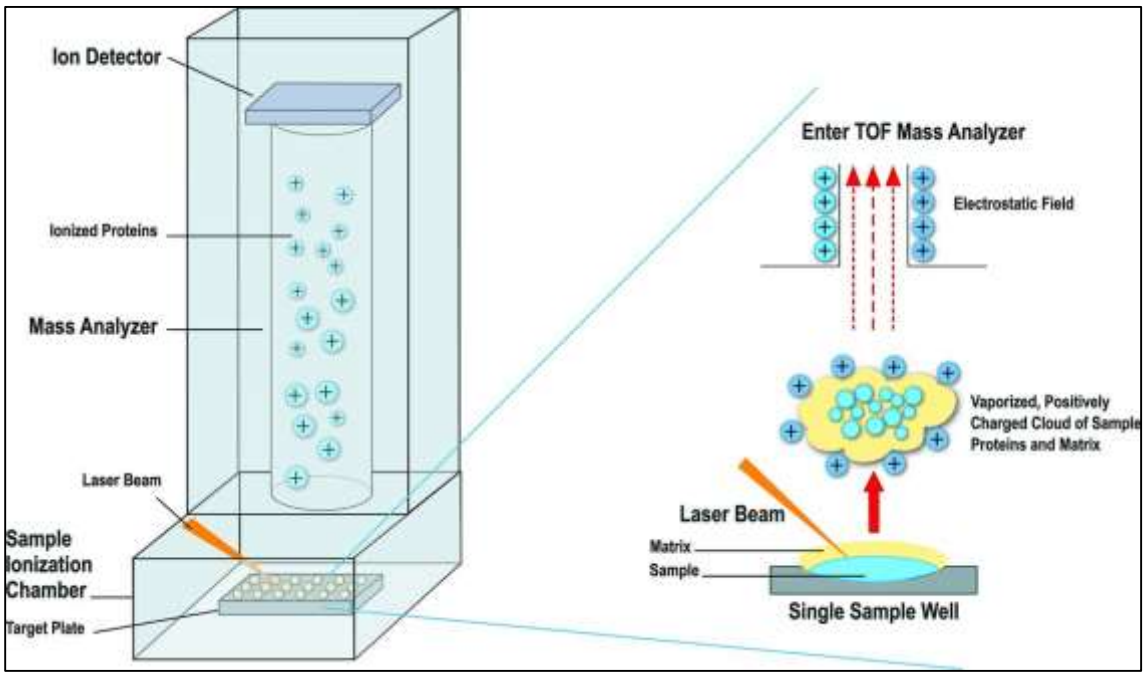

Figure 4. MALDI-TOF mass spectrometer. Using a plastic loop a colony is spreaded from a culture plate to a spot on a MALDI-TOF MS target slide. One or many isolates may be tested at a time. The spot is overlain with 1–2 µL of matrix and dried. The slide is placed in the ionization chamber of the mass spectrometer. Spots to be analyzed are shot by a laser, desorbing and ionizing microbial and matrix molecules from the target slide. The cloud of ionized molecules is accelerated into the TOF mass analyzer, toward a detector. A mass spectrum is generated and compared against a database of mass spectra by the software, resulting in identification of the organism (5).

3 Phenotypic methods of antimicrobial activity

Antimicrobial susceptibility testing (AST) of bacterial pathogens is one of the principal tasks of the clinical microbiology laboratory and phenotypic AST is still considered the gold standard for the determination of antimicrobial susceptibility. Phenotypic AST testing offers two advantages as compared to genotypic testing methods: it predicts not only the drug resistance but also drug susceptibility and permits to quantify the level of susceptibility of a bacterial isolate to individual antimicrobials (53).

However, using traditional approaches, the total turnaround time of current antibiotic resistance detection process for blood samples from BSI patients is longer than three days since it requires three overnight culture steps: blood culture, subculture and antimicrobial susceptibility test culture. Thus, currently susceptibility testing performed in a clinical microbiology laboratory requires a pure culture of the pathogen. Once isolates colonies from the pathogenic organism are available the bacteria inoculum is prepared and standardized prior to performing AST via disk diffusion or broth dilution methods. Clinical breakpoints for different antibiotics and bacteria are reviewed and updated annually by national organizations, such as Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility testing (EUCAST). Regardless, conventional antibiotic susceptibility tests methods take a couple of days to perform from the time the blood culture becomes positive. There is interest in developing antibiotic susceptibility tests methods for bacterial blood isolates that can generate data in a more clinically meaningful time frame (54).

3.1 Agar disk diffusion

The disk diffusion susceptibility method (55) is simple, practical and is well standardized. For routine susceptibility tests, the inoculum may be prepared by making a direct saline or broth suspension of colonies selected from an 18 to 24h nonselective nutrient agar plate and by applying a bacterial inoculum of approximately 1-2 $X10^8$ CFU/mL to the surface of a Mueller-Hinton agar plate. Commercially-prepared, fixed concentration, paper antibiotic disks are placed on the inoculated agar surface and plates are incubated for 16 – 24 h at 35°C prior to determination of results. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. Antibiogram provides qualitative results by categorizing bacteria as susceptible, intermediate or resistant. Since the bacterial growth inhibition does not mean the bacterial death, the agar disk-diffusion method is not appropriate to determine the minimum inhibitory concentration (MIC), as it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium (56).

In many clinical microbiology laboratories an agar disk diffusion method is routinely used for the testing of common, rapidly growing, and some fastidious bacterial pathogens, allowing categorization of most such isolates as susceptible, intermediate, or resistant to a wide range of antimicrobial agents. This approach is particularly common in resource limited settings and when performed according to standardized methods, such as those published by the CLSI, provides accurate direction to therapeutic antibiotic decisions.

There are several advantages to the disk diffusion approach to antibiotic susceptibility tests, including the following: (a) it is technically easy to perform and results are reproducible, (b) the reagents and supplies are inexpensive, (c) it does not require the use of expensive equipment, (d) it generates categorical interpretive results well understood by clinicians; (e) it allows for considerable flexibility in the selection of

antibiotics for testing; (f) allows visibility of growth, correct inoculum and mixed cultures; (g) It is the least costly of all susceptibility methods (57).

3.2 Antimicrobial gradient

The M.I.C. Evaluator (Oxoid, Cambridge, UK) and Etest (bioMérieux, Durham, NC), are commercially available gradient diffusion systems for quantitative antibiotic susceptibility tests. Both systems use preformed antimicrobial gradients applied to 1 face of a plastic strip to generate diffusion of drug into an agar-based medium. The assays are performed in a manner similar to that for disk diffusion using a suspension of test organism (fresh culture) equivalent in turbidity to that of a 0.5 McFarland. After overnight incubation, the tests are read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip. Generally, the results have correlated well with MICs generated by broth dilution methods. The gradient diffusion method has intrinsic flexibility by being able to test laboratory chosen drugs. However, Etest/M.I.C.E strips cost approximately R$ 10 – R$ 15 each and can represent an expensive approach if more than a few drugs are tested (58).

3.3 Broth Dilution - Minimum Inhibitory Concentration Testing

The miniaturization and mechanization of the broth dilution method by use of small, disposable, plastic “microdilution” trays have made broth dilution testing practical and popular. This procedure involved preparing two-fold dilutions of antibiotics in a liquid growth medium dispensed in standard trays contain 96 wells (Microdilution panels). Then, each well is inoculated with a microbial inoculum prepared in the same medium after dilution of standardized microbial suspension adjusted to 0.5 McFarland scale. MIC value recorded is defined as the lowest concentration of the assayed antimicrobial agent

that inhibits the visible growth of the microorganism tested, and it is usually expressed in µg/mL or mg/L. The precision of this method was considered to be plus or minus 1 two-fold concentration, due in large part to the practice of manually preparing serial dilutions of the antibiotics. The advantage of this technique is the generation of a quantitative result (MIC). The principal disadvantages of the microdilution method are the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test. Thus, a few clinical microbiology laboratories prepare their own panels (58).

Automated systems. Semi-automated devices of broth microdilution assay, which use optical systems to measure subtle changes in bacterial can standardize the reading of end points and often produce susceptibility test results in a shorter period than manual readings (6 - 12h), because sensitive optical detection systems allow detection of subtle changes in bacterial growth. The BD Phoenix Automated Microbiology System (BD Diagnostics) and The Vitek 2 System (bioMérieux, France) are the most common automated identification/AST systems currently used in Brazil. Compared to manual methods, these instruments provide a streamlined workflow and quantitative results, thus simplifying MIC determinations for pathogenic bacteria isolated from clinical samples. Nevertheless, these automates instruments still require the use of isolated bacteria grown in pure culture and the susceptibility tests are based on measuring bacterial growth and turbidity change. As a result, it remains inherently slow and limited by the low sensitivity of the current detection methods. Moreover, they are limited in the number of antibiotics and concentrations tested, lack the capability of analyzing polymicrobial samples or heterogeneous response of bacterial populations to antibiotics and very costly for a routine clinical laboratory (23, 59).

3.4 Emerging techniques for antibiotic susceptibility tests

In addition to rapid identification, administration of appropriate antibiotics is essential to improve patient care. Organism identification can guide antibiotic therapy; however, many organisms have unpredictable resistance patterns and need full AST to determine optimal treatment. Given the ever-increasing spread of antibiotic resistance, efforts to reduce the time-to-result for phenotypic AST are crucial to facilitate timely administration of appropriate antimicrobials (60) .

Many molecular assays have been developed to improve the turnaround time of blood cultures using PCR and microarray technologies. These assays employ a simple workflow, significantly reduce the turnaround time, may give genotypic resistance information, and are highly sensitive, leading to their adoption by many clinical laboratories. However, molecular methods require additional hands on processing time and the cost is often high for clinical laboratories, as an initial investment is necessary for the instruments (39).

With the introduction of new technologies and the modification of existing ones, it is possible to achieve microorganism identification and antimicrobial susceptibility profile from blood cultures within a short time frame (60). In principal, accelerating of classical phenotypic AST techniques is a strategy to meet this challenge in three possibilities: a) replacement of standardized inoculum prepared from pure culture by directly using positive blood cultures as a starting point for AST (direct AST); b) accelerating bacterial growth (short-term incubation) ; c) early reading of disc diffusion method (53).

Antimicrobial susceptibility testing directly from positive blood culture has been reported in multiple papers by performing directly AST via disk diffusion method (54); early reading of disc diffusion method (54, 61) and automated devices – Vitek 2 (22, 62-

71). Nevertheless, the American Society for Microbiology (ASM), the British Society for Antimicrobial Chemotherapy (BSAC) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) seriously criticize directly AST via disk diffusion method, since the inoculum is not standardized (22).

The short-term incubation protocol involves bacterial cultures in the log phase of growth (4-6 incubation) rather than in the stationary phase (16-24h incubation) and literature supports that the physiological phase of bacterial cell growth does not significantly influences of standardized disc diffusion AST (60, 72). Moreover, the short-term incubation protocol allows the inoculum standardization as recommended by AST standards.

4 Clinical impact of rapid microbiology tests for bloodstream infections

Sepsis presents the most substantial diagnostic and therapeutic challenge of all BSIs, although the term BSI can also refer to various grades of bacteremia. Bacteremia is defined as the presence of bacteria in the bloodstream and can be diagnosed as transient, intermittent, or continuous. When these circulating bacteria and their toxins elicit a dysregulated host response, resulting in significant organ dysfunction, circulatory collapse, and metabolic deterioration, sepsis, a true medical crisis, occurs. Understanding the burden placed on the healthcare system by sepsis is key to appreciating the need for rapid diagnosis of the causative organism(s) and their antimicrobial susceptibility. The most recent report on sepsis by the Agency for Healthcare Research and Quality revealed that sepsis-related hospital stays increased by 153% between 1993 and 2009, with an average annual increase of 6%. Sepsis is also the single-most expensive reason for hospitalization, with an annual cost estimated in excess of $ 20 billion (23, 24). In-hospital mortality rates from sepsis are a staggering 16%, over 8 times higher than other diagnoses, with as many as 600 deaths occurring per day in the United States alone.(73)

The pace at which new rapid diagnostic technologies, heralded as “game changers” by some in the infectious disease community, are evolving presents a challenge to infection prevention (IPs), whose role and responsibilities have already undergone a dramatic expansion. Maintaining a working knowledge of the basic principles of the different rapid methods and the information they provide; determining which technology best meets the needs and goals of their antimicrobial stewardship program (ASP) and infection prevention programs; and learning how they can advocate for the technology in their institution often requires time and resources that IPs no longer have. The use of broad-spectrum, empiric therapy in treating BSIs, including sepsis, has repeatedly been implicated as a contributor to antimicrobial resistance. Despite this, empiric therapy remains a mainstay of BSI — and particularly sepsis — treatment for several valid reasons. In fact, the international Surviving Sepsis Campaign Guidelines recommend “empiric broad-spectrum therapy with one or more antimicrobials for patients presenting with sepsis or septic shock to cover all likely pathogens (including bacterial and potentially fungal or viral coverage). This practice is based on the fact that, in many cases of primary BSIs, the clinical picture belies a specific microbiologic diagnosis, leading healthcare providers to initiate therapy that covers a broad range of potential pathogens. Additionally, the acuity of BSIs and the knowledge that mortality directly correlates with time to effective therapy precludes waiting for ID and AST results. Thus, the longer the turnaround time (TAT) for those results, the longer it takes to de-escalate therapy and the more likely the empiric therapy is to contribute to downstream resistance. A vicious cycle ensues in which suspicion of resistant organisms as causative pathogens in BSI leads to the use of increasingly broad-spectrum antibiotics. Rapid diagnostics represent a significant advance from traditional culture methods on the continuum of BSI diagnostic capabilities. Blood culture and traditional AST methods are still the core laboratory practice; however, they are increasingly being supplemented with novel

diagnostics that yield information hours to days faster than the traditional techniques. Most of these rapid diagnostics dramatically improved the time-to-result associated with identification of the most common bacteria and yeast that cause BSIs. Significantly, until early 2017, advances in time-to-result in new AST methods have generally lagged behind those for identification and resistance marker testing (73). In a study of MALDI-TOF-based rapid identification for BSI and Antimicrobial Stewardship Programmes interventions compared with conventional blood culture bacteria identification and no Antimicrobial Stewardship Programmes intervention, Huang et al. (3) demonstrated significantly reduced time to effective therapy (84.0 vs 55.9 hours, P<.001), mortality (20.3% vs 12.7%, P = .021), and length of intensive care unit stay (14.9 vs 8.3 days, P = .014) in the MALDI-TOF group. In two separate studies, Perez et al. (2) demonstrated similar results for the same MALDI-TOF ID system and Antimicrobial Stewardship Programmes interventions in Gram-negative bacteremia along with significantly reduced hospital expenditures (mean reduction of $ 19,547).

5 RELEVANCE OF THE STUDY

The development of rapid diagnostic methods is necessary for improving the diagnosis of bloodstream infections as well as assessing the antimicrobial susceptibility of the causative bacteria. This process contributes significantly for the early diagnosis and treatment of complex bloodstream infections and sepsis. MALDI-TOF is a new diagnostic toll developed for this purpose. A comparative analysis with traditional diagnostic methods in microbiology is essential for understanding its use in clinical laboratories.

6 AIMS OF THE STUDY

6.2 GENERAL AIM

To evaluate a modification of standard methodology in microbiology for the identification and antimicrobial susceptibility testing of bacteria in positive blood cultures in order to take advantage of MALDI TOF MS technique.

6.3 SPECIFIC AIMS

- To evaluate the performance of MALDI-TOF VITEK®MS System (bioMérieux, France) for bacteria identification after a short incubation on solid medium of positive blood cultures compared to our routinely used method of performing bacteria identification;
- To analyze the accuracy of rapid AST in comparison to gold standard method in Gram negative bacteria and Staphylococcus spp.

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8. MANUSCRIPT

INTEGRATING BACTERIAL IDENTIFICATION AND SUSCEPTIBILITY TESTING: A SIMPLE AND RAPID APPROACH TO REDUCE THE TURNAROUND TIME IN THE MANAGEMENT OF BLOOD CULTURES.

Running title:

Rapid modified bacterial identification and antimicrobial resistance detection from positive blood culture

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ABSTRACT

Background. Bloodstream infection is a critical disease with high mortality rate. Adequate treatment required rapid microbiological identification and timely administration of appropriate antibiotics. Rapid diagnostic tests that accurately identify infection-causing pathogens and the effective antimicrobials against these pathogens can increase the likelihood that patients are treated appropriately. Rapid diagnostic tests can also be used to help clinicians discontinue unnecessary antibiotics or de-escalate broad-spectrum antimicrobial therapy to a narrower-spectrum option. Current technologies employed in blood culture routine diagnostics are precise and sensitive but rather slow, most depending on bacterial growth. **Objective.** In this study, we evaluated a rapid bacterial identification (rID) and a rapid antimicrobial susceptibility testing by disk diffusion (rAST) from positive blood culture to overcome the limitations of the conventional methods. We investigated a rapid workflow to reduce the turnaround time in bloodstream infections diagnostics in a routine microbiology laboratory. **Methods**. The study included hemocultures flagged as positive by BACT/ALERT® (Biomérieux, Marcy l'Etoile, France) between 7-12:00 a.m. and inoculated on Chocolate agar at our clinical microbiology laboratory. At 16.00 p.m., after 4-6h incubation at 35-38°C (5% CO_2), identification by MALDI-TOF MS (VITEK MS® system, Biomérieux, Marcy-l'Étoile, France) and AST (disk diffusion method) were performed. Results were compared to identification (sID) and AST (sAST) results after 24h incubation. An identification score value of > 96% was considered as a correct species identification. For AST categorical agreement (CA), very major errors (VME, false-susceptible result of rapid AST), major errors (ME, false-resistant result of rapid AST), minor errors (mE, false categorization involving intermediate result) were investigated. **Results.** We identified a total of 526 bacterial isolated from blood cultures obtained from patients attended at a tertiary

hospital in the south of Brazil, 246 Gram-negative (GN) and 279 Gram-positive (GP) aerobes. The overall concordance between rID and sID was 88.6% and was highest for GN (95.5%). *K. pneumoniae* and *E. coli* presented 96.0% and 98.5% rate of concordance, respectively. Total of 2196 and 1476 antimicrobial agents' comparisons were obtained for GN and GP, respectively. Evaluating rAST, the CA, VME, ME and mE were 97.7, 0.7, 0.5 and 1.1% for GN and 98.0, 0.5, 0.7 and 0.8% for GP, respectively. Meropenem CA, VME and ME were 98.3, 0.5 and 0.5%, respectively; no mE was observed. Oxacillin CA, ME and mE were 97.4, 1.6 and 0.6%, respectively; no VME was observed. Sensitivity and specificity of rAST method were calculated for each antimicrobial agents. Meropenem presented 99.2 and 98.1% and Oxacillin presented 96.9 and 97.9% of sensibility and specificity, respectively. Overall Kappa scores of the comparisons results demonstrated the high agreement between rAST and sAST. **Conclusions.** The rapid methods for bacterial identification and for rapid AST results proposed in this study were distinguished from standards ones by feasible modifications and the accuracy of rAST were comparable with the standard method. Identification and AST of aerobic bacteria from positive blood cultures after a shortened incubation on solid blood agar is a fast and reliable method that may improve management of bloodstream infections, allowing us to save up to 24 h to identifying bacteria and supply useful information to adapt antibiotic therapy when necessary.

Keywords: blood culture; rapid identification; MALDI-TOF; rapid antimicrobial resistance detection; disk diffusion

INTRODUCTION

The development of rapid diagnostic assays for the identification and analysis of antimicrobial resistance of bacterial causing bloodstream infections is of utmost importance to reduce morbidity and mortality. Infectious diseases have a substantial global health impact. Fast diagnosis of pathogens is critical to guarantee the most adequate therapy for infections (1). Bloodstream infections can be caused by a wide variety of microorganisms, commonly *E. coli*, *Klebsiella* spp., *S. aureus*, other bacteria and yeast. Rapid identification of bloodstream pathogens is a laboratory practice that supports rapid transitions to direct target therapy, supporting timely and effective patient care (2). Current technologies employed in routine diagnostics are based on bacterial culture, which constitute the actual gold standard, are precise and sensitive but rather slow. Traditional identification and antimicrobial susceptibility test results for microorganisms causing bloodstream infections can take 48h or longer to obtain. Today, new methods have been made available to enable faster diagnosis. Recently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) emerged as a rapid, accurate, cost-effective method and has been effectively used as a rapid method for identifying a wide array of microbial species (3). In MALDI-TOF MS analysis, abundant structural proteins such as ribosomal proteins are extracted from an intact bacterial colony. The ionizing laser vaporizes structural proteins of microorganisms, and unique mass spectra are generated, having mass-to-charge ratio (m/z) peaks with varying intensities. The mass spectra of test isolates are sequentially compared with those in a reference database for identification (4). In comparison with conventional methods for identification of clinical samples by and despite its high-end technology, a MALDI-TOF MS device is simple to use. MALDI-TOF MS can provide advantages for a universal procedure of microbial identification. Only a small amount of an organism, typically a fraction of a single colony from primary culture plates, is required

for analysis. Comparatively, a larger inoculum and subculture is often required for conventional biochemical methods or other automated systems. Furthermore, once the instrument is loaded, identifications can typically be performed in less than one minute, compared with hours to days for conventional methods (4, 5).

Even simpler and faster than traditional bacterial identification methods, analysis of blood cultures via MALDI-TOF MS and the detection of antimicrobial resistance requires a preliminary bacterial culture in solid media. In this study, we evaluated a rapid modified bacterial identification and a rapid antimicrobial resistance detection from positive blood cultures based on centrifugation and short-time bacterial incubation to reduce the turnaround time in bloodstream infections diagnostics in a routine microbiology laboratory.

MATERIALS AND METHODS

Study design and study population

Transversal study. All gram negative bacilli and *Staphylococcus* sp. isolates recovered from bloodstream infection during 2017 to 2018 of patients in treatment at Hospital de Clínicas de Porto Alegre.

Blood culture procedures

Blood culture sets were obtained from *patients* attended at a tertiary hospital in south of Brazil. Aerobic and anaerobic blood culture bottles (bacT/ALERT® culture media FA Plus, PF plus and FN Plus, bioMérieux, France), were forwarded to the Microbiology Unit and incubated in the automated blood culture system bacT/ALERT® 3D (bioMérieux). Negative *bottles* are automatically resulted and discarded after *5 days'*

incubation. The performance methods were evaluated for positive blood cultures with monomicrobial bacterial growth. Bottles yielding polymicrobial or yeast growth were excluded. For analyzes, we considered only one blood culture series per individual patient. Moreover, the testing only included blood cultures from the daily microbiology laboratorial routine, no artificially inoculated vials were evaluated in this study.

Standard bacterial identification (sID) and antimicrobial susceptibility testing (sAST) from positive blood culture

When bacT/ALERT®3D system flagged a blood culture as positive it was analyzed by Gram staining followed by subculture on an appropriate solid agar medium (aerobic Columbia agar 5% Sheep Blood, Chocolate agar, MacConkey agar, bioMérieux, France) following 18-24 h incubation at 35°C and 5% CO_2 atmosphere. The colonies grown on overnight agar plates incubation were spotted onto a target slide, prepared according to the manufacturer's instructions for analysis using VITEK MS® system software version 3.0 (bioMérieux), which was termed standard identification method (sID). The colonies grown on overnight agar plates incubation were also used for inoculum disk diffusion test preparation, according to CLSI (6), which was termed standard disk diffusion method (sAST). The bacterial identification and antimicrobial resistance detection results obtained using this conventional workflow were used for comparison in the data analyses.

Rapid modified bacterial Identification (rID) from positive blood culture

The rapid modified bacterial identification from positive blood culture evaluated was previously described by Chen Y et al. (2015) (7). Briefly, 3 mL of positive blood culture broth were aspirated from positive blood culture bottles using a sterile syringe and transferred to a 10 mL serum separator tube. The aspirate was centrifuged for 5 min

at 3,000 rpm, the supernatant was discarded and 20 µL of the bacterial pellet was transferred to the center of a Chocolate agar plate (bioMérieux). The inoculum was streaked out to form a 2 by 2 cm, the plate was incubated at 35°C in 5% CO_2 atmosphere, for up to 4-6 h. Growth on the plate was recovered with a 1 µL inoculating loop, to obtain a sufficient inoculum to be spotted onto a target slide prepared, according to the manufacturer's instructions for analyzes using MALDI-TOF VITEK MS® system software version 3.0 (bioMérieux). The identification process was performed only once for each sample. Criteria for interpretation of the results proposed by the manufacturer was a single organism identification (same genus and specie) as successful identification, and more than one species results with the same genus, as acceptable identification.

Rapid modified antimicrobial susceptibility testing (rAST) from positive blood culture

A modification of the standard disk diffusion method (rAST) was evaluated to detection of antimicrobial resistance. The rAST followed CLSI standards (6) in all aspects, with exception by the inoculum preparation. CLSI guideline´s recommended to prepare the inoculum for AST by direct suspension of isolated colonies selected from 18-24 h agar plate incubation. In this study we used colonies selected from rapid culture grown (4-6h agar plate incubation). The inoculum was prepared from the rapid culture grown on a 150mm Mueller Hinton Agar (bioMèrieux, France) and then, disks were applied and plates incubated at 35°C±1° and incubated for 18h. The following antimicrobial agents were evaluated for GN: Amikacin 30 µg, Amoxacillin-clavulanate 20/10 µg, Ampicillin 10 µg, Ampicillin-sulbactam 10/10 µg, Cefepime 30 µg, Ceftazidime 30 µg, Cefuroxime 30 µg, Ciprofloxacin 5 µg, Gentamicin 10 µg, Meropenem 10 µg, Piperacillin-tazobactam 100/10 µg and Trimethoprim- sulfamethoxazole 23,75/1,25µg (Oxoid®, Thermo-Fisher, USA). For GP Cefoxitin 30 µg, Clarithromycin 15 µg,

Clindamycin 2 µg, Doxycyclin 30 µg, Erytromycin 15 µg, Gentamicin 10 µg, Levofloxacin 5 µg, Rifampicin 5 µg and Trimethoprim- sulfamethoxazole 23,75/1,25 µg (Oxoid®) were evaluated. The inhibition zones were analyzed after 18-24 h and the results were interpreted by CLSI proposed breakpoints (6).

Data analysis

Bacterial identification and antimicrobial resistance detection results obtained by the rapid modified methods were compared with those obtained by standard methods. Bacterial identification results were classified as correct identification at species or genus levels and non-reliable identification and the success rate of rID was calculated.

Sensitive (S), intermediate (I) and resistant (R) interpretative results were evaluated for each antimicrobial agent tested by disk diffusion methods, and categorical agreement (CA) between rAST and current standard method was determined. Categorical discrepancies were classified as a very major error (VME), or a false-susceptible result; a major error (ME), or a false-resistant result; and a minor error (mE) when one method yielding an intermediate result and the other yielding a susceptible or resistant result. The acceptable inter-method categorical discrepancies rates of VME, ME and mE are ≤1.5%, ≤3% and ≤10%, respectively (8).

Statistical analysis

The concordance of antimicrobial resistance results was determined using the categorical agreement and discrepancies rates for the detection of antimicrobial resistance with 95% confidence intervals (CI). The 95% CIs for the proportion of categorical agreement between the rAST and the sAST, including VME, ME and mE, were also calculated. Kappa coefficients were calculated using a 95% CI. Kappa

interpretation: < 0 Less than chance agreement; 0.01–0.20 Slight agreement; 0.21– 0.40 Fair agreement; 0.41–0.60 Moderate agreement; 0.61–0.80 Substantial agreement; 0.81–0.99 Almost perfect agreement. All statistical analyses were performed using SPSS *Versions* 20.0.

RESULTS

In total 524 bacterial isolated from blood cultures, 246 GN and 277 GP were included. The overall bacterial concordance rate of rID was 88.6% and highest rate was observed for GN (95.5%). *K. pneumoniae* and *E. coli* presented of 96.0% and 98.5% of concordance, respectively. *P. aeruginosa* and *A. baumannii* Complex presented a 100.0% and 92.9% of bacterial identification concordance, respectively. *S. aureus* presented 98.6% and Coagulase-negative *Staphylococci* (CNS) presented the lower rate of concordance in rID method (77.7%) (Table 1).

A total of 2196 and 1476 antimicrobial agent's susceptibility testing comparisons were obtained for GN and GP, respectively. The overall CA, VME, ME and mE were 97.7, 0.7, 0.5 and 1.1% for GN and 98.0, 0.5, 0.7 and 0.8% for GP, respectively (Table 2 and 3). When we analyzed these categorical discrepancies by antimicrobial agents used for GN, we observed that all antimicrobial agents presented acceptable limits of discrepancy, with the exception of Piperacillin-tazobactam VME value (2.2%). Meropenem CA, VME and ME were 98.3, 0.5 and 0.5%, respectively; and no mE was observed (Table 2). When we analyzed these categorical discrepancies by antimicrobial agents used for GP, we observed that all antimicrobial agents presented acceptable limits, with the exception of Clarithromycin VME value (1.8%) and Levofloxacin ME value (3.7%). Oxacillin CA, ME and mE were 97.4, 1.2 and 0.6%, respectively; and no VME was observed (Table 3).

When we analyzed these categorical discrepancies by bacterial species, we observed that all bacteria species presented acceptable limits. *Enterobacteriales* group presented a CA, VME, ME and mE of 97.4, 0.4, 0.3 and 1.4%, respectively. *K. pneumoniae* and *E. coli* presented a CA, VME, ME and mE of 99.1, 0.4%, 0.2% and 0.6% and 97.1, 0.3, 0.2 and 2.5%, respectively. *P. aeruginosa* presented a CA of 97.9, a VME of 1.2%, and no ME and mE detection. Staphylococci group presented a CA ≥97% *Staphylococcus aureus* and Coagulase-negative *Staphylococci* (CNS) presented a CA, VME, ME and mE of 99.1, 0.1, 0.3% and 0%, and 97.2, 0.2%, 0.5% and 0.7% respectively (Table 4).

Sensitivity and specificity of rAST were calculated for each antimicrobial agents used for GN and GP. We observed that all antimicrobial agents used for GN and GP presented high values of sensibility and specificity. Meropenem presented 99.2 and 98.1% of sensibility and specificity, respectively (Table 5). The lower sensibility for GN was for Ampicillin (84.2%) and specificity was for Ciprofloxacin (96.2%). Oxacillin presented 96.9 and 97.9% of sensibility and specificity, respectively. The lower sensibility for GP was for Clarithromycin (94.5%) and specificity was for Doxycycline (84.6%) (Table 6).

Kappa scores of the 2196 comparisons results of antimicrobial resistance detection for GN bacteria isolates (n=183) were determined k= 0.85 to 0.99 (Table 7). Kappa scores of the results of antimicrobial resistance detection for *K. pneumoniae* (n=73) isolates (Table 8) and for *E.coli* (n=54) isolates (Table 9) were from 0.91 to 0.97 for both. Kappa scores of the 1476 comparisons results of antimicrobial resistance detection were obtained for GP bacteria isolates (n=164) and the values were from 0.87 to 0.99. The overall Kappa score *p* value was < 0.001.

DISCUSSION

Rapid Identification and antimicrobial susceptibility testing for bacterial causing bloodstream infections is of utmost importance to reduce morbidity and mortality. Infectious diseases have a substantial global health impact. Bloodstream infection is still among the ten most common causes of deaths in developed countries (9). Incidence rates between 80 and 257 per 100,000 person-year have been reported, with higher rates in the more recent years (10, 11), and the case fatality rate constitutes 10 - 40% (12, 13). In spite of great treatment efforts during the last decades, infections are still among the most common causes of death worldwide. In the last decades, a rising occurrence of microorganisms resistant to antimicrobial agents is of particular concern (14).

Current technologies employed in routine diagnostics are based on bacterial culture, which constitute the actual gold standard, are precise and sensitive tests but rather slow. We modified previously developed bacterial identification and antimicrobial susceptibility testing methods from blood culture to propose a faster, easier and reliable method to reduce turnaround time of bloodstream infection diagnosis in a routine microbiology laboratory. The proposed method in this study were distinguished from standards ones by centrifugation and short-time grown on solid media.

The analyzes of the correct identification at species or genus levels and the success rate of de rapid identification methods were calculated. The correct bacterial identification rate was higher among GN isolates. The clinical relevance of nosocomial Enterobacteriaceae, as *K. pneumoniae*, and non-fermentation Gram-negative as *P. aeruginosa*, identification from blood culture in a short time leads to an impact in bloodstream infections management, especially in ITU patients. The CNS presented the lower correct bacteria identification, mainly at species level. The clinical impact of CNS

identification at species level require caution, considering the doubtful of this bacteria group as causing bloodstream infections or as a blood collection contaminant (15).

Multiple methods of direct identification from positive blood cultures have been proposed with correct identification to the species level ranging from 67% to 93% (16-21). Commonly found with these published procedures is the ability to correctly identify Gram-negative bacteria more frequently than Gram-positive bacteria, a finding we also encountered. However, the procedures that have been employed are often laborious, typically requiring an extraction procedure with centrifugation, lysis, or filtration of the specimen. Advantages of our protocol include its simplicity and speed, requiring only 5 to 10 min for preparation of the smudge plate. Results are available within up to 4-6 h, and the procedure is easily incorporated into routine microbiology laboratory workflow.

In our study, interpretative results of disk diffusion methods were evaluated for each antimicrobial agent tested by rAST. We obtained lower rates of VME, ME and mE for GN and GP, thus rASt presented acceptable values according to intermethods rates (FDA). When we analyzed these categorical discrepancies by antimicrobial agents used for GN, we observed that only Piperacillin-tazobactam presented VME value (2.2%). For GP only Clarithromycin VME value (1.8%) and Levofloxacin ME value (3.7%). Overall Kappa scores of the comparisons results of rAST and sAST demonstrated the concordance between the two methods.

In conclusion, the success rate of rID, CA, Kappa coefficient and the accuracy of rAST were accepted to be adopted in the routine since the inter-method error rates were above those acceptable (8). The rapidity and reliability were factors in its adoption for routine use, allowing us to save up to 24 h in identifying bacteria from blood culture and supplying useful information to adapt antibiotic therapy when necessary.

Faster diagnosis of pathogens is critical to guarantee the most adequate therapy for infections. Delay in the initiation of appropriate antibiotic therapy has been recognized

as a risk factor for mortality. Ferrer R et. Al (2014) evidenced significant association between delay in antibiotic administration over the first 6 hours after identification of patients with severe sepsis and septic shock and increasing mortality. These results underscore the importance of early identification and treatment of septic patients in the hospital setting. As mentioned often in the literature, sepsis is a time-dependent condition and should be recognized as an urgent situation that requires immediate response (22). Empirical treatment with broad-spectrum antibiotics is started in patients suspected of bacteremia; however, diagnostics are necessary, as recent studies have observed that 25 to 33% of patients are inappropriately treated within the first 24 h due to lack of coverage from organism's resistant to broad-spectrum antibiotics. To improve patient care, rapid bacterial identification and susceptibility results for blood cultures are necessary (23).

Additionally, rapid and accurate antimicrobial susceptibility test is paramount to the management of patients with serious infections. The ability to report identification and susceptibility results from positive blood cultures shortly after they signaled positive for growth is of great value in reducing time to appropriate therapy (4, 24). Otherwise, the current culture-based AST tools rely on time-consuming culturing techniques, followed by disk-diffusion and broth dilution susceptibility testing. In many clinical microbiology laboratories, agar disk diffusion is routinely used, while automated AST instruments are limited in the number of antibiotics, concentrations tested and lack the capability of analyzing polymicrobial samples or heterogeneous response of bacterial populations to the antibiotics (25).

In the light of ever increasing problems related to the emergence of multidrug-resistant bacteria, rapid microbiological diagnostics are of growing importance. Timely pathogen detection and availability of susceptibility data are essential for optimal treatment, but are even more crucial for de-escalation of broad

spectrum empiric therapies. Considering what represent the gain of 24 h in the turnaround time of bloodstream infection diagnosis in a nosocomial routine microbiology laboratory concerning morbidity, mortality, antimicrobial resistance development, ITU, economical costs among others, we conclude that this method is feasible for use in routine microbiology laboratories.

Identification and susceptibility by rapid phenotypic methods shows a high degree of accuracy; the marked reduction in time to results may have significant implications for patient care.

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TABLE 1. Identification performance of rapid bacterial identification (rID) versus the standard bacterial identification method. Concordance rate for species-level and genus-level and non-reliable identification by Vitek MS system.

Organism ID by current standard method	N (%) Identified in Vitek MS (rID)			
		Concordance level		
	n	Species	Genus	No ID
Overall	524	459 (87.3)	466 (88.6)	58 (11)
Gram-negative bacteria	246	235 (96)	235 (96)	11 (4)
K. pneumoniae	100	96 (96)	96 (96)	4 (4)
E. coli	67	66 (99)	66 (99)	1 (1)
K. oxytoca	15	13 (87)	13 (87)	2 (13)
E. cloacae	6	6 (100)	6 (100)	0
P. mirabilis	5	5 (100)	5 (100)	0
E. hormaechei	4	4 (100)	4 (100)	0
S. marcescens	3	3 (100)	3 (100)	0
C. koseri	2	1(50)	1 (50)	1 (50)
M. morganii	1	1(100)	1 (100)	0
P. aeruginosa	22	22 (100)	22 (100)	0
A. baumannii	14	13 (93)	13 (93)	1 (7)
B. cepacia	4	3 (75)	3 (75)	1 (25)
S. maltophilia	3	3 (100)	3 (100)	0
Gram-positive bacteria				
Staphylococci	278	224 (81)	231(83)	47 (17)
S. aureus	73	72 (99)	72 (99)	1 (1)
Coagulase-negative Staphylococci	205	153 (75)	160 (78)	45 (22)

TABLE 2. AST performance of rAST compared with standard method. Categorical agreement (CA), very major errors (VME), major errors (ME) and minor errors (mE) per antibiotic agents used for Gram-negative (GN) and Gram-positive (GP). Kappa scores of the 2196 antimicrobial agents determinations result of rAST for Gram-negative bacteria isolates and 1476 antimicrobial agents determinations result of rAST for Gram-positive bacteria isolates.

Antimicrobial Agents	CA (%)	VME (%)	ME (%)	mE (%)	Kappa score

GN					
Amikacin	98.3	1 (0.5)	1(0.5)	0(0)	0.96
Amoxilicin-clavulanate	98.1	0 (0)	1(0.5)	2(1.1)	0.95
Ampicillin	97.0	0(0)	1(0.5)	4(2.2)	0.85
Ampicillin-sulbactam	97.8	0(0)	1(0.5)	8(4.4)	0.93
Cefepime	96.9	2 (1.1)	1(0.5)	1(0.5)	0.97
Ceftazidime	97.2	1 (0.5)	1(0.5)	2(1.1)	0.96
Cefuroxime	97.2	1(0.5)	1(0.5)	2(1.1)	0.93
Ciprofloxacin	97.8	1(0.5)	1(0.5)	1(0.5)	0.94
Gentamicin	97.5	2(1.1)	1(0.5)	0(0)	0.95
Meropenem	98.3	1(0.5)	1(0.5)	0(0)	0.97
Piperacilin-tazobactam	93.0	4(2.2)	1(0.5)	5(2.7)	0.90
Trimethoprim-sulphametoxazole	98.3	2(1.1)	0(0)	0(0)	0.99
Total	97.7	15(0.7)	10(0.5)	25(1.1)	
GP					
Clarithromycin	95.5	3 (1.8)	1(0.6)	1(0.6)	0.91
Erytromycin	99.0	0 (0)	1(0.6)	0 (0)	0.99
Clindamycin	98.0	2 (1.2)	0 (0)	0 (0)	0.87
Doxycyclin	100	0 (0)	0 (0)	0 (0)	0.91
Rifampicin	100	0 (0)	0 (0)	0 (0)	0.99
Gentamicin	96.6	1 (0.6)	0 (0)	4 (2.4)	0.99
Levofloxacin	91.9	1(0.6)	6 (3.7)	2 91.2)	0.91
Oxacillin	97.4	0 (0)	2 (1.2)	1(0.6)	0.95
Trimethoprim-sulphametoxazole	95.6	0 (0)	2 (1.2)	4 (2.4)	0.91
Total	98	7(0.5)	11(0.7)	12(0.8)	

9. FINAL CONSIDERATIONS

The purpose of this thesis was to modify previously developed bacterial identification and antimicrobial resistance detection methods from blood culture to propose a faster, easier and reliable method reducing the turnaround time of bloodstream infection diagnosis in a routine microbiology laboratory. The proposed methods in this study were distinguished from standards ones by centrifugation and short-time grown on solid media.

The growing rate of use for MALDI-TOF MS in clinical settings is currently paving the way for a high throughput, cost-effective approach to replace the laborious and time consuming traditional methods. In this sense the optimization of blood culture routine at microbiology laboratories are now possible and reliable by integration of MALDI-TOF MS for bacteria identification and antibiotic susceptibility techniques.

The key points from the study are:

- The sample preparation for bacteria identification by MALDI-TOF MS does not require extraction. In other words, it is a simple protocol and feasible for routine practice with appropriate accuracy. No additional reagents or training of laboratory staff is required.
- The identification concordance observed between the rapid and standard method was higher for BGN (95.5%) than for *Staphylococcus* sp. isolates (88%).
- High CA agreement ≥ 97% was observed between the rapid and standard method. Meropenem CA was 98.3% for *K. pneumoniae* isolates and Oxacillin CA was 97.4% for *Staphylococcus* sp.

- A short incubation in solid media directly from positive blood cultures allowed for both a rapid species identification and an antimicrobial susceptibility result nearly 24h earlier than is possible applying the standard methodology.

The promising results from the database analysis proved that the assays are simple and cost-effective methods that can be implemented in a routine microbiology laboratory. Decreasing turnaround time is a hot topic in microbiology and in particular for blood culture diagnosis. Direct identification of bacteria from blood cultures with MALDI-TOF is already implemented in some routine laboratories. However, often, no information regarding antibiotic (non)-susceptibility of the bacteria is reported.

10. FUTURE WORK

Early diagnosis and initiation of appropriate antibiotic treatment together with earlier de-escalation of broad-spectrum antibiotics is critical to improve patient outcome. Therefore, integration of rapid identification and susceptibility techniques with antimicrobial stewardship significantly could improve time to optimal therapy, and it could decrease hospital length of stay and total cost in our institution. Additional validation and a study of the impact on mortality and morbidity would be useful in the future. Thus, interdisciplinary collaborative study will provide an important framework for productively addressing many other clinical care problems.

However, further studies are necessary to confirm the reliability of this method with more pathogens with different resistance phenotypes. Moreover, integration of additional techniques as rapid detection of carbapenem-resistant *Enterobacteriaceae* by

MALDI-TOF MS approach would be useful, and will provide additional information to the clinical staff.

11. ATTACHMENT

STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	43
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	44
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	45-47
Objectives	3	State specific objectives, including any prespecified hypotheses	47
Methods			
Study design	4	Present key elements of study design early in the paper	47
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	47
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	47
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	47-49
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of	48-50

		assessment methods if there is more than one group	
Bias	9	Describe any efforts to address potential sources of bias	18
Study size	10	Explain how the study size was arrived at	45
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	NA
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	50
		(*b*) Describe any methods used to examine subgroups and interactions	
		(*c*) Explain how missing data were addressed	
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	
		(e) Describe any sensitivity analyses	50
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analyzed	**51**
		(b) Give reasons for non-participation at each stage	**NA**
		(c) Consider use of a flow diagram	**NA**
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and	**51**

		information on exposures and potential confounders	
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers of outcome events or summary measures	
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	**49-51**
		(*b*) Report category boundaries when continuous variables were categorized	**49-51**
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	**49-51**
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	**49-51**
Discussion			
Key results	18	Summarise key results with reference to study objectives	**52-58**
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	**52-58**
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations,	**52-58**

		multiplicity of analyses, results from similar studies, and other relevant evidence	
Generalisability	21	Discuss the generalisability (external validity) of the study results	**52-58**
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	**58**