

FAST: hastening antimicrobial susceptibility testing

David José Silva Abreu

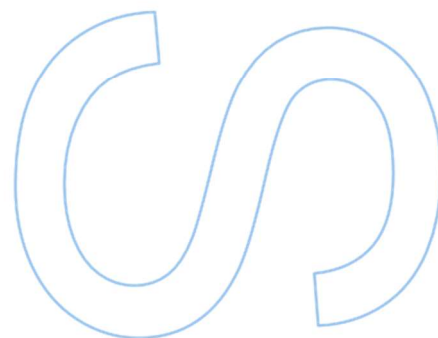
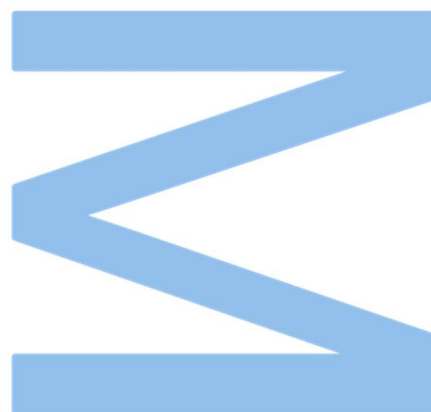
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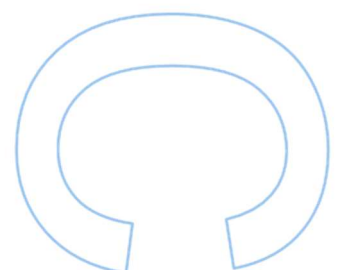
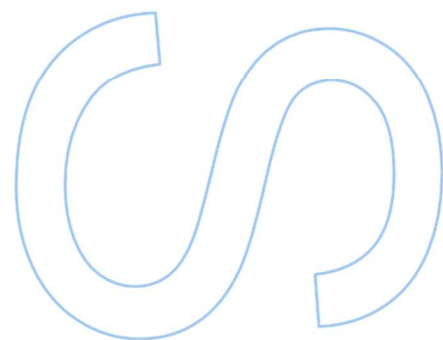
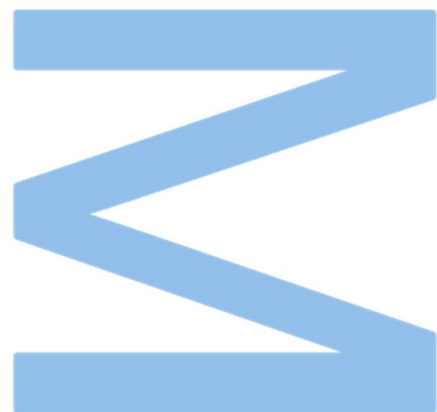
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David José Silva Abreu

Porto, 29 de setembro de 2022

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Resumo

Os agentes antimicrobianos são uma importante mais-valia no tratamento de infeções, sendo assim utilizados em larga escala mundialmente. No entanto, devido a mecanismos genéticos bacterianos, a exposição a agentes antimicrobianos está a resultar no aparecimento de estirpes com resistência ao seu mecanismo de ação, negando o seu efeito terapêutico. A resistência a antimicrobianos está a aumentar progressivamente com o aumento do seu uso terapêutico, ao ponto de ser reconhecida atualmente como uma das maiores ameaças à saúde humana.

Um possível atenuante do problema seria o melhoramento da cadeia de procedimentos envolvidos no diagnóstico de doenças infecciosas, especialmente as que são comumente associadas a agentes patogénicos resistentes e acompanhadas de elevada taxa de mortalidade agravada pela demora do início da terapia, tais como as infeções da corrente sanguínea e sépsis. O diagnóstico consiste em vários procedimentos morosos tais como hemoculturas, identificação bacteriana e testes de suscetibilidade a antimicrobianos, que culminam num elevado tempo até à produção de resultados, que costuma demorar vários dias. A introdução de alternativas de diagnóstico mais rápidas poderá adiantar a transição da terapia com antibióticos de largo espectro para uma terapia direcionada, reduzindo o peso que estas infeções exercem nos serviços de saúde, e especialmente no aparecimento da resistência a antimicrobianos. Desta forma, surgiram vários métodos rápidos de identificação bacteriana e especialmente de testes de suscetibilidade, tendo-se verificado o estabelecimento de um nicho de mercado composto por várias empresas que oferecem a sua solução para o problema, algumas das quais foram discutidas neste trabalho.

Uma destas empresas, a FASTinov, desenvolveu um teste de suscetibilidade a antimicrobianos baseado em citometria de fluxo de fluorescência que gera resultados em 2 horas, tornando-o no teste mais rápido no mercado. Neste trabalho, a performance de dois kits de suscetibilidade—FASTgrampos e FASTgramneg—foram testados tendo em conta métodos de referência (microdiluição em caldo e teste de difusão em discos), utilizando hemoculturas inoculadas com isolados clínicos e estirpes de referência. Os resultados gerais estavam em concordância com os padrões estabelecidos para métodos de diagnóstico de suscetibilidade (concordância essencial e categórica $\geq 90\%$; percentagem de erros muito graves e percentagem de erros graves $\leq 3.0\%$), à exceção da percentagem de erros graves total obtida para isolados gram-positivos, que excedeu ligeiramente o limite (3.2%) considerando as concentrações limite estabelecidas pelo

EUCAST, facto que poderá ter sido devido a um pequeno tamanho de amostra. Desta forma, o método foi considerado apto, tal como já tinha sido verificado em validações prévias dos produtos.

Além disso, um estudo preliminar da exatidão de dois métodos de identificação rápida por ionização por dessorção a laser assistida por matriz com analisador por tempo de voo diretamente através de hemoculturas, um deles desenvolvido pela FASTinov, foi realizado de modo a avaliar a sua inclusão no protocolo de procedimento dos produtos da empresa. A percentagem de exatidão total do método FASTinov (83.13%) foi maior do que a obtida para o método baseado em dodecil sulfato de sódio (67.47%). Além do mais, a percentagem de identificações erradas observada em ambos os métodos foi muito baixa, com apenas um caso identificado no método de dodecil sulfato de sódio. Embora tenha consistido apenas num estudo preliminar, os resultados obtidos indicam a potencial utilidade que os métodos rápidos de identificação podem ter quando incorporados em protocolos de testes de suscetibilidade rápidos.

Além do trabalho laboratorial, o estágio na FASTinov permitiu-me contactar e participar em alguns aspetos comerciais da empresa, tendo por isso sido considerado por mim como uma experiência de enorme valor, do ponto de vista pessoal e académico.

Palavras-chave: infeções da corrente sanguínea, sépsis, resistência a antimicrobianos, testes de suscetibilidade a antimicrobianos, citometria de fluxo, identificação de bactérias, espetrometria de massa

Summary

Antimicrobials are an important asset in the treatment of infections and as such are widely used. However, due to bacterial genetic mechanisms, exposure to antimicrobials is conferring strains resistance to their mechanism of action, negating their therapeutic effect. Antimicrobial resistance progressively increased with the increased use of antimicrobial therapy, to the point that it is now recognized as one of the biggest threats to human health.

One possible solution to deescalate the issue is the improvement of the chain of procedures for diagnosis of infections, which is especially relevant in conditions such as bloodstream infections and sepsis, which have an associated high mortality that is aggravated with the time until effective therapy is initiated, and that are highly associated with resistant pathogens. Diagnosis consists of several time-consuming procedures such as blood culturing, bacterial identification, and antimicrobial susceptibility testing which culminate in a very long time to results, taking several days. The introduction of less time-consuming procedures might allow for an early shift from broad-spectrum to narrower-spectrum antibiotic therapy, reducing the burden of these infections in healthcare, and especially in the spread of antimicrobial resistance. As such, several rapid methods of bacterial identification and susceptibility testing have emerged, with special emphasis for the latter, which has truly established a market niche with several companies offering their solution to the issue, some of which were discussed in this work.

One of these companies, FASTinov, has developed an antimicrobial susceptibility test based on fluorescence flow cytometry that provides results in 2 hours, currently the fastest in the market. In this work, the performance of two of FASTinov's susceptibility kits—FASTgrampos and FASTgramneg—was assessed against reference methods (broth microdilution and disk-diffusion test), using blood cultures spiked with clinical isolates and reference strains. The obtained overall results were in agreement with the standards for novel susceptibility diagnostic methods (Categorical and Essential Agreement percentages $\geq 90\%$; very major error percentage and major error percentage $\leq 3.0\%$), with the exception of the obtained total major error percentage for gram-positive isolates, which slightly exceeded the limit (3.2%) considering the established EUCAST breakpoint concentrations, which might have been due to a small sample size. As such, the performance of the method was considered to be apt, as was previously observed in other validations of the kits.

In addition, a preliminary study assessing the accuracy of two rapid identification methods by matrix absorption laser deionization – time of flight mass spectrometry directly from blood cultures, one of which developed by FASTinov, was performed in order to evaluate the possibility of incorporation of rapid identification in the workflow of FASTinov's products. The obtained overall percent accuracy of the FASTinov method (83.13%) was higher than the sodium dodecyl sulphate-based method (67.47%). In addition, the percentage of misidentifications observed on both methods was very low, with only one case being identified in the sodium dodecyl sulphate method. Although it was a preliminary study, the obtained results indicate the potential usefulness that rapid identification methods can have while implemented in rapid susceptibility testing protocols.

Besides laboratory work, the internship at FASTinov has allowed me to participate and help in some commercial aspects of the company, for which I considered it an invaluable experience, from a personal and academic standpoint.

Keywords: bloodstream infections, sepsis, antimicrobial resistance, antimicrobial susceptibility testing, flow cytometry, bacterial identification, mass spectrometry

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Abbreviation list

AST	- Antimicrobial Susceptibility Test
ATCC	– American Type Culture Collection
BHI	– Brain Heart Infusion
CA	– Categorical agreement
CAMHB	– Cation-adjusted Mueller-Hinton Broth
CCUG	– Culture Collection University of Gothenburg
CFU	– Colony Forming Units
CHUSJ	– Centro Hospitalar Universitário de São João
CLSI	– Clinical and Laboratory Standards Institute
CNPC	– Congresso Nacional de Patologia Clínica
DALY	– disability-adjusted life years
DiBAC ₄ (3)	– <i>Bis</i> -(1,3-dibutyl-barbituric acid) trimethine oxonol
EA	– Essential agreement
ECCMID	– European Congress for Clinical Microbiology and Infectious Diseases
EUCAST	– European Committee on Antimicrobial Susceptibility Testing
FAST	– Flow Cytometry Antimicrobial Susceptibility Testing
FDA	– Food and Drug Administration
FISH	– fluorescence <i>in-situ</i> hybridization
GBD	– Global Burden of Diseases, Injuries and Risk Factor study
HCCA	– α -cyano-4-hydroxycinnamic acid
HGT	– Horizontal Gene Transfer
I	– “Intermediate”/”Susceptible, increase exposure” (susceptibility category)
ID	– identification
ISO	– International Organization for Standardization

IVD – *In vitro* Diagnosis

IVDR – *In vitro* Diagnosis Regulation

ME – Major errors

mE – minor errors

MGE – mobile genetic elements

MHA – Mueller-Hinton Agar

MIC – Minimum inhibitory concentration

MRSA – Methicillin-resistant *Staphylococcus aureus*

PA – Percent accuracy

PCR – polymerase chain reaction

PI – propidium iodide

R – “Resistant” (susceptibility category)

rAST – Rapid Antimicrobial Susceptibility Test

RPM – revolutions per minute

S – “Susceptible” (susceptibility category)

SDS – sodium dodecyl sulphate

SDD – “Susceptible, dose-dependent” (susceptibility category)

VME – Very major errors

Introduction

1. Antimicrobial Resistance

1.1. Antimicrobials and mechanisms of action

An antimicrobial is a pharmaceutical drug that is able to kill or inhibit the growth of susceptible microorganisms. Antimicrobials permit the clearance of bacterial, fungal, viral or parasitic infections through selective toxicity. Antibiotics, which are antimicrobials that present inhibitory or lethal effects specifically against bacteria, were the main focus of this work [1].

Different types of antimicrobials can present different mechanisms of action, with the inhibition of cell wall synthesis being one of them. β -lactam antibiotics, such as penicillin, are able to bind to bacterial transpeptidases (also called penicillin-binding proteins), interfering with the final stage of synthesis of peptidoglycan, the main component of the bacterial cell wall. Susceptible bacteria exposed to β -lactams are therefore vulnerable to cell lysis and consequently death during growth, conferring bactericidal potential to β -lactam antibiotics, which remain one of the most relevant antibiotic classes [2].

Some antibiotics can inhibit the function of the cell membrane, such as polymyxins. These are antibiotics that bind with high affinity to lipopolysaccharides present in the outer membrane of gram-negative bacteria, leading to the destabilization of these molecules and resulting in loss of integrity of the outer membrane. The consequent permeability increase allows for the uptake of the polymyxin into the periplasm, which proceeds to disrupt the inner membrane. Polymyxin E, or colistin, is used as a last-line antibiotic against gram-negative bacteria [3]. Daptomycin is a lipopeptide antibiotic used against gram-positive pathogens that also affects membrane function through the induction of depolarization, however, the exact mechanism is not yet fully understood [4].

The inhibition of protein synthesis is also the mechanism of action of some antimicrobials which target bacterial ribosomes in a selective manner, as these highly differ from mammalian ribosomes in composition. Aminoglycosides such as streptomycin bind to the 30S subunit of bacterial ribosomes and cause the misreading of the template mRNA strand, leading to mistranslated proteins and consequent cell death [5].

An exploitable mechanism of action is also the inhibition of nucleic acid synthesis, characteristic of quinolones and fluoroquinolones, which target topoisomerase enzymes. Ciprofloxacin is a widely used broad-spectrum fluoroquinolone used against both gram-

positive and gram-negative pathogens that binds to subunit A of DNA gyrase, inhibiting its activity and interfering with DNA replication, leading to cell death [6].

These mechanisms of action explain the usefulness of antimicrobial drugs as therapeutical agents for infections, contributing to the improvement of the clinical outcome of the host through the clearance of the bacterial pathogen. However, due to the nature of their genetics, bacteria can present resistance to the mechanism of action of certain antibiotics, rendering their therapeutical effect as suboptimal or even fully negating it.

1.2. Antimicrobial resistance – definition and mechanisms of resistance

Antimicrobial resistance (AMR) is characterized by the ability of a microorganism to circumvent the mechanism of action of a single or multiple antimicrobial drugs. The usage of the broader “antimicrobial” term includes fungi and viruses that present resistance to antimicrobial drugs. However, the discussion in this work was reserved primarily for antibiotic resistance.

The most relevant mechanisms of resistance are usually a consequence of the expression of AMR genes, which lead to the translation of proteins that counter the mechanism of action of an antimicrobial through many aspects. There are several possible mechanisms of resistance, in which are included the destruction/alteration of the antimicrobial agent itself, alterations in the antimicrobial's target, and reduction in the accumulation of the antimicrobial in the cell. Considering the mechanism of action of one of the most utilized antibiotic classes, β -lactams, examples for several possible mechanisms of resistance were provided onward.

Destruction of the antimicrobial itself can be exemplified by β -lactamase mediated β -lactam resistance. β -lactamases are enzymes that can hydrolyse the β -lactam amide, impeding the antimicrobial's usual mode of action of binding to penicillin-binding proteins, as this is dependent on an intact β -lactam ring. The PC1 enzyme, coded by the *blaZ* gene, is an example of a clinically relevant β -lactamase that conferred resistance to penicillin in *Staphylococcus aureus*, which ultimately led to the introduction of methicillin as an alternative treatment, as it was not affected by β -lactamases [7].

Another possible resistance mechanism relies in efflux pumps, which are transporter proteins present in the cell membrane that mediate the uptake of several different substrates, including antibiotic agents. An example is the OprD porin, which is responsible for the uptake of carbapenems, a class of β -lactam antibiotics, in

Pseudomonas aeruginosa. Mutations in the *oprD* gene can influence permeability to carbapenems, culminating in the reduced uptake of these antibiotics and thus the development of a resistant phenotype [8]. Efflux pumps can be extremely specific for a single substrate or have a very wide range of different substrates, and as such can have a role in the emergence of multi drug-resistance phenotypes [9].

1.3. Historical contextualization of AMR

AMR was first described in 1940, when members of the penicillin discovery team identified a bacterial penicillinase capable of inactivating the drug, even before its introduction as a therapeutic agent. As penicillin became widely used as an antibiotic, resistant strains started becoming more prevalent, and thus began the need to develop synthetic variants of penicillin and novel antimicrobials which retained their effectiveness against the resistant strains [10].

Despite the effectiveness of the newly introduced antimicrobials however, bacteria were able to quickly adapt, as with every new drug introduced, additional resistant strains were being identified. In response to the penicillin-resistant strains, methicillin was introduced in 1959, and just a year later a methicillin-resistance *Staphylococcus* strain was identified [11]. As methicillin-resistance *Staphylococcus aureus* (MRSA) strains became pandemic, the clinical usage of vancomycin increased as these strains initially retained susceptibility to this antimicrobial agent. This led to the appearance of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus* strains (VISA and VRSA, respectively) [12]. This summarizes a very relevant problem associated with antimicrobial treatment: resistance to it will emerge, sooner or later [13]. This game of “cat and mouse” has prevailed throughout the years and led to the appearance of many resistant phenotypes (Figure 1).

The production of novel antimicrobials was considered adequate during a period of time referred to as the antimicrobial discovery “golden era”, which occurred approximately from the 1940s until the 1980s, depending on the sources [11, 14, 15]. Natural antimicrobial compounds were being isolated from producing bacteria, mainly from soil samples, and new antimicrobial classes were being discovered almost in a yearly basis. From 1980 onwards however, novel antimicrobial production stagnated which has led to the fact that the vast majority of antimicrobial classes in use still today were introduced during the golden era. In addition, these were isolated from a limited number of ecological niches and taxonomic groups, as 64% of natural antimicrobial classes are produced by filamentous actinomycetes [14, 16]. As further exploration of this niche with

contemporary technology did not lead to the discovery of new antimicrobial classes, the modern approach to combat emerging resistance has been the modification of existing antimicrobials [14].

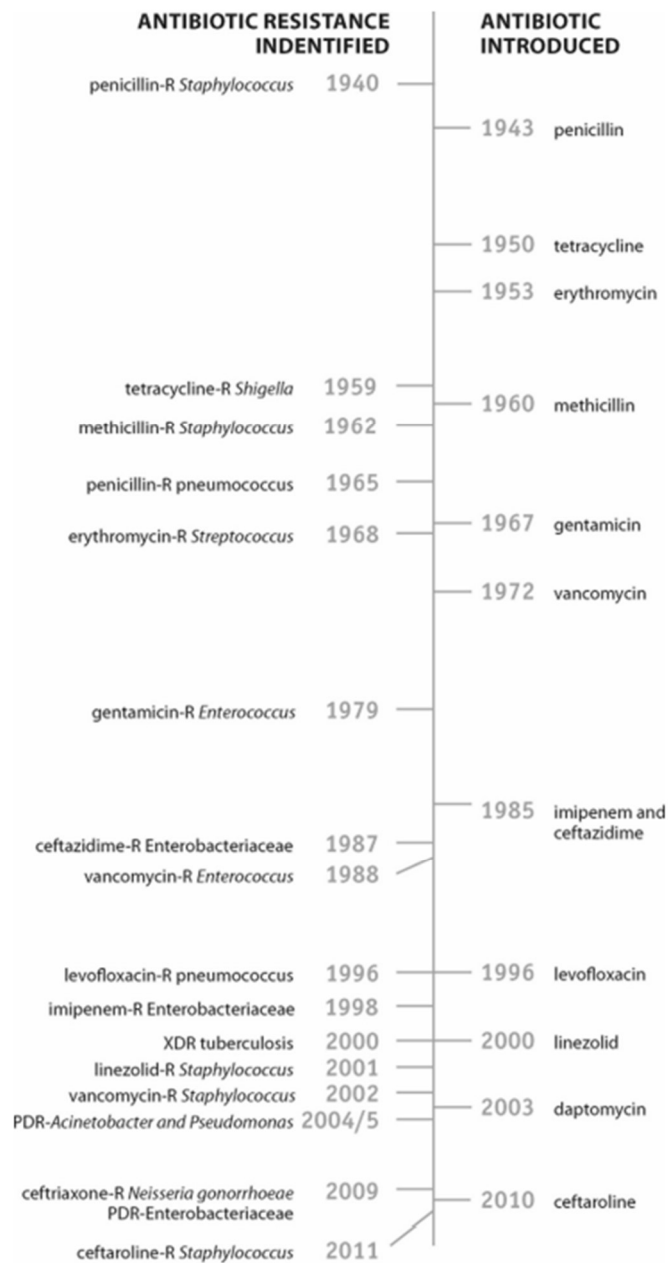


Figure 1: Timeline comparison of the introduction of several antibiotic agents with the respective reports of resistant strain. Withdrawn from (Ventola, 2015) [17].

As the research for new antimicrobial agents became increasingly harder, it also became less attractive from an economic standpoint, which is reflected on the fact that 10 out of

the 15 largest pharmaceutical companies have significantly reduced or abandoned efforts in novel antimicrobial research since 1999. This summarizes another challenging problem associated with antimicrobial treatment: AMR is increasing at a faster pace than new antimicrobials are being introduced [13].

The aforementioned challenges contribute to the progressive loss of effectiveness of antimicrobial therapy, leading to the escalation of AMR, which is currently considered a global threat of far-reaching proportions [18].

1.4. The healthcare burden of AMR

A recent systematic analysis assessing 88 pathogen-drug combinations, has concluded that the global fatal burden associated with drug-resistance infections in 2019 was 4.95 million deaths, of which 1.27 million deaths were directly attributed to drug resistance. This indicates that, in the best-case scenario, AMR is the 12th leading Global Burden of Diseases, Injuries and Risk Factor study (GBD) level 3 cause of death, being ahead of HIV and malaria. Besides deaths, the rate of global disability-adjusted life years (DALY) per 100 000 population associated with and directly attributed with AMR are also very impactful, being 2477.7 and 618.7, respectively [19].

Taking into account these numbers, it is understandable why AMR is consistently referenced by the World Health Organization as one of the biggest threats to human health, having secured a spot in the top 10 global public health threats to humanity, as well as in the 10 global health issues to track in 2021 [20, 21]. According to a report to the Secretary-General of the United Nations published by the World Health Organization in 2019, the World Bank estimated that the number of deaths associated with AMR per year can increase to 10 million in 2050, exceeding the fatal burden of cancer [22].

To prevent the escalation of this issue, the understanding of its origin and cause is necessary.

1.5. Causes of AMR

1.5.1. Bacterial genetics and acquired resistance

AMR is considered intrinsic if it does not involve antibiotic exposure and is associated with a certain bacteria group or species. For example, the *Pseudomonas* genus is intrinsically resistant to the large-spectrum antibiotic triclosan, due to being innately able to encode a resistant isozyme of the enzyme targeted by the mechanism of action of this

antibiotic [23]. However, a resistance mechanism can be acquired by mutations in the existing genetic material of a previously susceptible bacterium or the acquisition of new genetic material from an exogenous source, which constitutes acquired resistance [11].

The most common causes of the acquirement of AMR in bacteria are of genetic origin. As prokaryotes, bacteria do not have a differentiated nucleus encapsulating their DNA, having instead a single, haploid, circular, double-stranded chromosome that can be condensed into a structure—the nucleoid. As with every living organism, bacterial DNA can be vertically inherited i.e., replicated and passed on from parent to daughter cells. Due to their haploidy and lack of sexual reproduction, the only source of genetic diversity in bacteria would otherwise be the occurrence of mutations and their fixation in the descendency through vertical gene transfer. However, bacteria can also transfer genetic material to other pre-existing mature cells through a process called horizontal gene transfer (HGT) (Figure 2) [1].

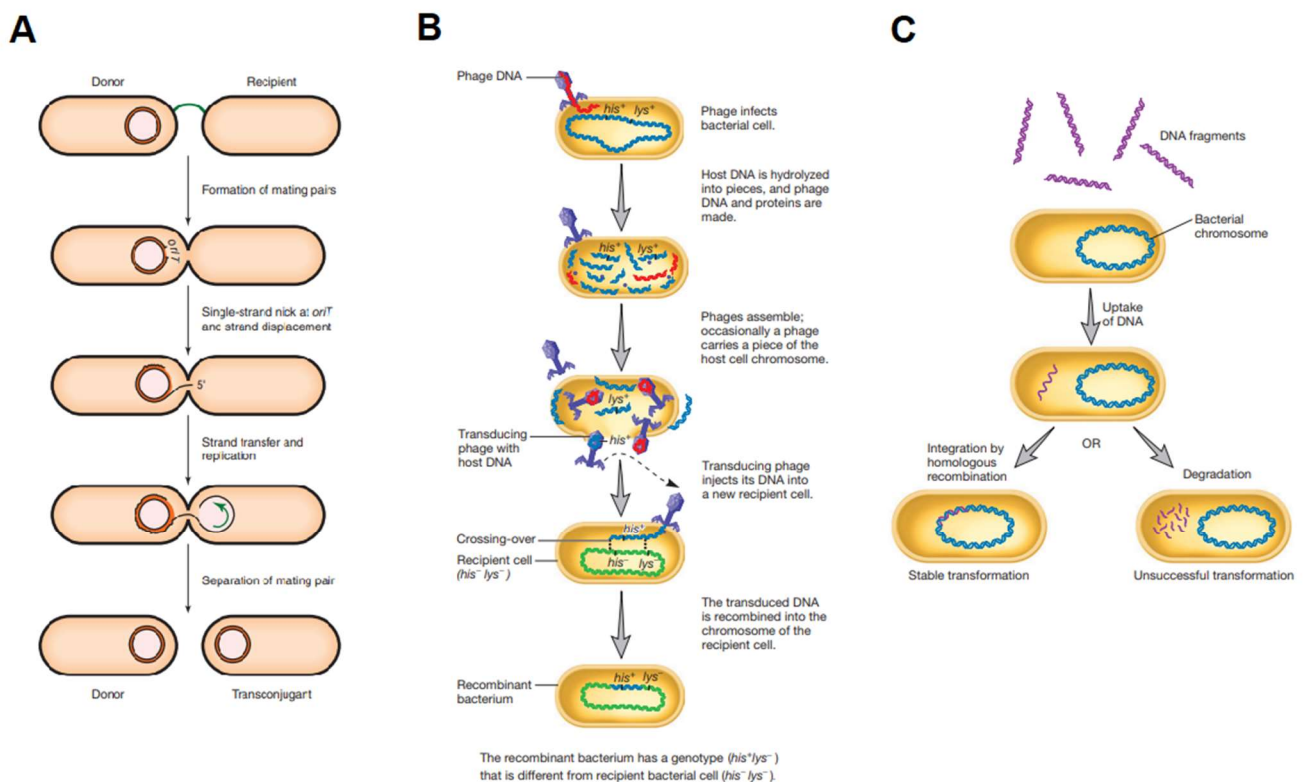


Figure 2: Schematic representation of several Horizontal Gene Transfer (HGT) mechanisms. A – Plasmid transfer through conjugation; B – Generalized transduction in bacteria; C – Transformation with DNA fragments. A withdrawn from *Jawetz, Melnick and Adelberg's Medical Microbiology*, 28th Edition (Riedel et al. 2019) [1]; B and C withdrawn from *Prescott's Microbiology*, 10th Edition (Wiley et al., 2017) [24].

1.5.2. HGT mechanisms and the spread of AMR

HGT involves mobile genetic elements (MGE), which promote the intra- and intercellular mobility of genetic information [25]. Although chromosomal AMR can occur by spontaneous mutation, the transfer of extrachromosomal MGE by HGT is of particular relevance to acquired AMR. HGT encompasses three main mechanisms—conjugation, transduction, and transformation—which were briefly discussed onward, along with their contributions to the spread of AMR genes.

1.5.2.1. Conjugation

Plasmids are self-replicating small molecules of circular extrachromosomal DNA that carry specialized genes. Besides being vertically transferred, plasmids also mediate conjugation, which appears to be the most important HGT mechanism for the transfer of resistance.

Conjugation consists in the transfer of a conjugative genetic element from one cell to another through direct contact between the cells, mediated by a sex pilus (Figure 2A). Conjugative elements can exist in the form of plasmids or as integrative conjugative elements, which integrate into the chromosome of the host bacterium. Conjugative elements contain genes responsible for the conjugation mechanism to occur and can also carry AMR mechanism genes associated with insertion sequences, transposons, integrons and other MGE, which when transferred to another cell are able to confer it a resistant phenotype that can be rapidly disseminated in a population of bacteria [26]. This dissemination can even occur to bacteria of different species from the donor if plasmids with a broad host range are involved.

The presence of multiple resistance genes in the same plasmid can induce resistance to several antimicrobial agents in a single conjugation event. This can contribute to the emergence of multidrug resistant bacteria, which are becoming increasingly common and are now endemic to several parts of the world. Infections by multidrug resistant pathogens very concerning as they are difficult to treat, as the pool of available antibiotics that can be considered as treatment options is small [11].

From an evolutionary biology perspective, the acquisition of a plasmid by a bacterium generally incurs in a fitness cost when in the absence of selection for traits associated with the obtained plasmid, which would limit the spread of the plasmid among a population [26]. As such, the spread of plasmids carrying AMR genes is favoured by positive selection associated with antimicrobial presence, which can explain the increasing pattern of AMR spread associated with antimicrobial exposure [27]. Besides

contributing to the maintenance of transferred plasmids, antimicrobial exposure can contribute to an increase in conjugation rate in a laboratory setting, although the clinical relevancy of the induced conjugative activity is disputed [28]. These observations seem to be in agreement with the finding that the presence of AMR genes in plasmids was not commonplace before the antibiotic era, underlining the role that antimicrobial exposure has had and continues to have in the spread of AMR [11].

1.5.2.2. Transduction

Transduction is acknowledged as a potential contributor to the intraspecies spread of AMR genes. This HGT process is mediated by bacteriophages, viruses that infect bacteria (Figure 2B). After encapsulating, bacteriophages can retain fragments of genetic information of the host bacterium, which can then be transferred and integrated into the chromosome of recipient bacteria as the bacteriophage infection spreads. Both chromosomal and plasmid DNA are transferrable through this mechanism, and exposure to antibiotics is likely to increase the rates of transduction [28].

Transduction was found to be relevant to the transfer of AMR genes in a laboratory setting, both in gram-positive and gram-negative bacteria. For example, the transfer of resistance to methicillin, penicillin, and tetracycline by transduction in *S. aureus* was observed in a laboratory setting [28, 29]. Bacteriophages were also found to play a role in the intra- and interspecies transfer of resistance to tetracycline and gentamicin in enterococci, through transduction of the *tetM* and *ant2-1* genes, respectively [30].

It is also hypothesized that the transfer of a resistance gene for β -lactam antibiotics through transduction was responsible for the emergence of gram-negative pathogens with resistance to this antibiotic class. Similar cases are likely to exist but have yet to be identified, possibly indicating the underappreciation of transduction in the spread of AMR genes [28].

1.5.2.3. Transformation

Transformation is the uptake of extracellular DNA by a competent cell, which can then be incorporated into the chromosome (Figure 2C). Although competence can be artificially induced in a laboratory setting, the discussion in this section will be reserved for competence occurring in a natural setting.

Although over 80 species of Gram-positive and Gram-negative bacteria include competence-related genes in their genome, not every individual cell enters the state, as the expression of such genes is usually not constitutive, requiring induction by factors such as high cell density, antibiotic stress, DNA damage, among others [31]. The

expression of these genes leads to the synthesis of 20-50 proteins that were found to regulate the state of competence, some of them being directly involved in DNA-uptake machinery, which can be species-dependent and is not yet fully characterized [31, 32].

Although natural competence for transformation is unusual, some data might suggest that it is an underappreciated method of HGT, as it can be very relevant to the transfer of plasmids between unrelated species [33]. It was verified that transformation rates can be increased by antimicrobial presence in a laboratory setting, which might indicate that exposure to antimicrobials is a potential contributor to the spread of resistance genes through this mechanism of HGT [28].

1.6. Managing AMR

Despite the beneficial uses of antimicrobial agents, it is very well established that there is a relationship between antimicrobial consumption and the emergence and spread of resistant pathogens [17]. While resistance seems to be an inevitable consequence of the use of antimicrobials, it is hypothesized that the issue is further escalated with overuse.

The overprescribing of antimicrobials appears to be a worldwide phenomenon. In some states of the US, the number of prescribed treatments exceeds the number of the population, and in Europe 35% of hospitalized patients were undergoing antibiotic therapy in 2011 [17, 34]. Misprescribing is also an issue, as incorrect use exposes patients to potential complications associated with antibiotic therapy, resulting in questionable therapeutic benefit [35]. Between 30 to 60% of antibiotics prescribed in intensive care units were considered unnecessary, inappropriate, or suboptimal [36].

Overuse in hospital settings is of particular importance. Clinical environments are especially prone to be highly selective for the emergence and transmission of resistant pathogens, as there is close proximity between frequently immunocompromised antimicrobial consumers which are undergoing intensive and prolonged treatment regimens [37].

Additionally, antimicrobials are still available without the need for prescription in some European Union member states for example and can be easily acquired by the general population [13]. Moreover, many studies indicate a general lack of knowledge of the population about antibiotics, leading to lack of adherence to the recommended therapy duration, or self-medication, including cases where antibiotics are wrongly used to treat flu-like symptoms or common colds [34].

Overuse does not seem to be limited to human communities or clinical environments, as antibiotic use is widespread in animals, agriculture, and aquaculture. The presence of antimicrobials in soil, water and sewage can contribute to the appearance of resistant pathogens. The recognition of the environmental presence of antimicrobial agents suggests that a one-health approach can be beneficial to better address AMR in a coordinated and multisectoral manner [38].

In summary, the primary way of combating AMR seems to be the promotion of prudent antimicrobial use, for which many strategies can be employed. Infection control and prevention, the altering of prescribing practices and the sensibilization of the population can be viable strategies to manage AMR emergence [37].

In clinical settings specifically, the establishment of antimicrobial stewardship programs, which are coordinated efforts to optimize antibiotic drug choice, dosage, duration of therapy and route of administration, can improve the appropriate use of antibiotic therapy in healthcare units. The accurate diagnosis of infectious diseases, especially in the case of infections with frequently unclear sources such as sepsis and septic shock, can avoid inappropriate therapy with empirical broad-spectrum antibiotics [39]. As clinical microbiology techniques are generally slow and work intensive, the improvement of diagnosis procedures through the rapid identification of the pathogen and the fast and accurate assessment of its susceptibility to antimicrobials can allow the clinician to optimize the therapeutic benefit of the treatment, by providing an earlier shift to targeted therapy with narrow-spectrum antibiotics [40].

In order to better explain the importance of rapid identification and antimicrobial susceptibility testing in promoting antimicrobial stewardship and de-escalating antimicrobial usage, as well as to provide a context to this internship at FASTinov, an overview of the diagnosis procedure of bloodstream infections and sepsis was discussed onward.

2. Bloodstream Infections and Sepsis

Bloodstream infection (BSI) is a condition defined by the presence of a viable pathogen in the blood of a host, able to be detected by blood culture. If the infection is of bacterial origin, the term bacteremia can also be used to refer to the condition [41]. BSI is associated with a high mortality rate, ranging from 14% to 37%, with the upper range being highly associated with intensive care unit hospitalizations [42]. Bloodstream infections are the main cause of morbidity and mortality in hospitalized patients [43]

In addition to the high risk of mortality, this condition is also relevant from the AMR standpoint, as the most recent global burden review on the subject has placed BSI as the infectious syndrome with the second highest number of deaths associated with and directly attributable to AMR, just under 1 500 000 and approximately 400 000 deaths, respectively (Figure 3) [19].

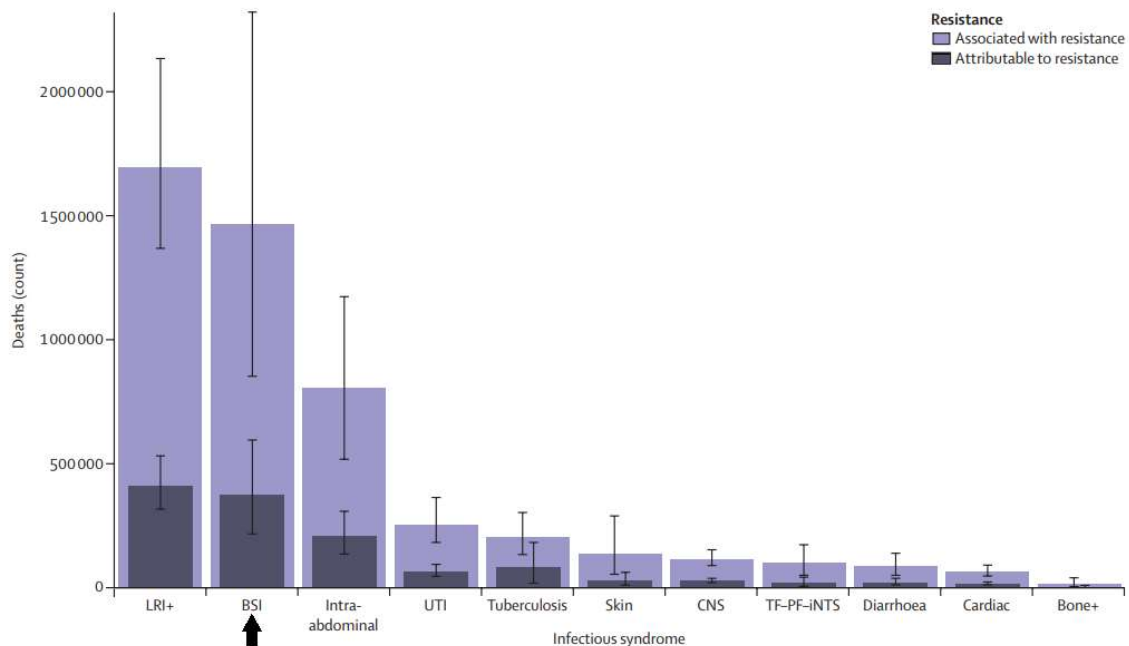


Figure 3: Global death counts attributable to and associated with AMR, per infectious syndrome. Black arrow indicating BSI, the second infectious syndrome with most death counts. Withdrawn from (Murray et al., 2022) [19].

Both gram-negative and gram-positive bacteria can be the causative agents for BSI. Although there is a historical higher prevalence of gram-negative bacteria on the aetiology of this condition, BSI caused by gram-positive bacteria are becoming increasingly common. Potential reasons for this include the increase in use of long-term indwelling catheters or changes in regimen of antimicrobial administration and prophylaxis [44].

The immune response to infection is triggered by endotoxins (cell wall lipopolysaccharides) in the case of gram-negative pathogens, or lipoteichoic acid, peptidoglycan, and extracellular toxins in the case of gram-positive pathogens. If the infection isn't cleared and the host response is prolonged, its pro-inflammatory and anti-inflammatory responses can induce detrimental effects, contributing to a highly dysfunctional inflammatory response, culminating in organ dysfunction and susceptibility to secondary infections [45]. This leads to sepsis, which is currently defined as life-threatening organ dysfunction caused by a dysregulated immune response to infection [46].

Sepsis is a severe condition with a very heavy burden on healthcare. A 2016 meta-analysis has estimated an incidence of 20 million cases per year, with a mortality of approximately 26%, potentially accounting for 5 million deaths worldwide [47]. Therapy consists in stabilization of blood pressure and antimicrobial prescription, which should start as soon as possible. If not adequately treated, the condition can progress to septic shock, which is defined by the current consensus as a condition in which the patient presents persistent hypotension refractory to fluid resuscitation, requiring vasopressors to maintain a mean arterial pressure superior to 65 mm Hg and hyperlactatemia represented by a serum lactate level of over 2 mmol/L, culminating in a higher than 40% mortality rate [46, 48]. Early recognition of sepsis is essential, as a 7.6% increase in mortality rate is observed for every hour of delay in antimicrobial therapy after the recognition of hypotension in the patient. The timely initiation of adequate antimicrobial therapy is of utmost importance, as it is the strongest predictor of clinical outcome in sepsis patients [49].

The high mortality rate of BSI and sepsis, the impact in outcome associated with a delay in antimicrobial therapy, and the escalation of these conditions due to AMR underline the importance of precise and fast diagnosis of these diseases, as well as a robust standard of care.

2.1. BSI/Sepsis diagnosis overview

Clinical microbiology and bacteriology in particular have been evolving at a slow pace, as classical 19th century technology is still commonplace in microbiology laboratories today. Growth-dependent microbiology is still the basis of many procedures, such as microbe detection, identification, and antimicrobial susceptibility testing [50]. This is especially impactful in the diagnosis and management of BSI/sepsis, as these conditions require fast detection for timely initiation of therapy due to the associated high mortality

rate. This, however, is still affected by very slow and growth-dependent diagnosis procedures, which culminate in a 48-72 hours turnaround time between the detection of a positive blood culture and the production of the antimicrobial susceptibility report (Figure 4) [51].

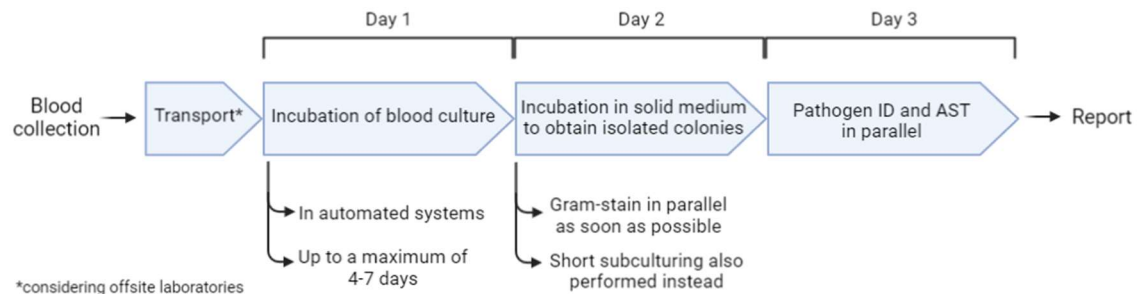


Figure 4: Schematic representation of the classical approach on BSI/sepsis diagnosis, including timeframes of the performed procedures. Timeframe not to scale for illustration purposes. ID – Identification; AST – Antimicrobial Susceptibility Testing. Illustration designed on BioRender [52], and adapted from (Lamy et al., 2020)[53].

Due to the importance of diagnosis and management of BSI/sepsis patients, international guidelines were established with several recommendations for clinicians, through the Surviving Sepsis Campaign, in order to standardize preferred sepsis treatment [54]. For this reason and to facilitate the evaluation of the clinical outcome at specific moments, sepsis “bundles of treatment”, which are sets of diagnostic and therapeutic manoeuvres performed at a specific time, were introduced. The 1-hour bundle places importance in the collection of blood cultures, which is the first step of the sepsis diagnosis procedure, right before the initiation of empirical antimicrobial therapy to control the infection [55]. Due to the importance of antibiotic therapy in sepsis treatment, antimicrobial stewardship programs, which were referenced before in this work, are also recommended, as these have shown to optimize clinical outcome while minimizing the downsides of antimicrobial use, such as toxicity and emergence of AMR [56].

In order to emphasize the need for faster diagnostic methods as a means to improve clinical outcome and AMR management, the standard diagnostic procedure for BSI and sepsis was discussed onward, starting with blood culture collection.

2.1.1. Blood cultures

Blood cultures remain the standard first line tool for the detection of BSI and sepsis. Routine blood cultures are performed in adult patients with suspected BSI as sets of two bottles, aerobic and anaerobic, ideally prior to the initiation of antimicrobial therapy

(Figure 5). The following of guidelines for proper blood collection is essential, as there are many factors associated with this step which can influence the correct diagnosis of bacteremia.



Figure 5: Representation of the BD Bactec Aerobic and Anaerobic Standard blood culture media bottles. Withdrawn from the products' webpages in the company's website [57, 58].

According to several available guidelines, the optimal number of collected bottle sets is between two and three, and these should generally be obtained from peripheral blood from different venipuncture sites with adequate aseptic technique, in order to avoid false-positives due to contamination by skin microbiota. Additionally, the volume of collected blood must be sufficient, and should be between 8 and 10 mL per bottle to avoid false-negative results [45, 53].

After collection, blood culture bottles are incubated at 35°C until positive or for a maximum of 5 to 7 days. Incubation occurs in continuous-monitoring automated systems, which have largely replace manual and semi-automated systems [45]. The two most widely used systems are Bactec (Beckton-Dickinson) and BacT/ALERT Virtuo (bioMérieux), which detect the presence of microorganisms through the detection of colorimetric or fluorescence changes generated by a pH shift in culture media. This pH shift is due to CO₂ production by growing organisms, which consume the carbohydrate substrate present in the bottle culture media [59].

Despite being considered the most effective means of BSI detection, some publications infer a very low positivity rate of blood cultures which, in most settings, is between 5% to 13%. In addition, although there is a general agreement in recommendations across several available guidelines, the recommended procedures for blood collection are not always followed in some clinical settings [59]. As such, false-positives are still prone to occur due to contamination, with a reported 20% to 56% proportion of contaminants, and with one publication claiming that only 4% to 7% of blood cultures are true-positives [42, 59].

Some alternative methods have emerged to counter the very long time of detection of blood cultures. However, these are still considered suboptimal [53]. As such, blood cultures remain the gold standard method for the detection of BSI and the infectious source of sepsis, despite their limitations.

2.1.2. Pathogen ID

The first step of processing a positive blood culture is the Gram stain. It should be performed as soon as possible to quickly categorize the pathogen and initiate empirical antimicrobial therapy, which should ideally start within the first hour of diagnosis [45]. Before rapid methods were introduced, the detected pathogen was cultured in agar to evaluate growth and then subject to simple strain characterization techniques such as catalase testing or motility assessments, leading to preliminary microbial identification (ID). Further biochemical and/or enzymatic techniques could then be performed to complete species assessment, while antimicrobial susceptibility testing was performed simultaneously, in order to transition antimicrobial therapy from empirical to targeted. These procedures were dependent on bacterial growth, taking approximately 2 days from the positive flagging of a blood culture to the obtainment of results, and were manual or semi-automated. This led to the need of development of faster, growth-independent, more accurate and precise methods of ID [50, 60].

Nucleic acid-based methods have been widely used as an alternative to classical ID methods. Gene sequencing of highly conserved genes in bacterial species, specifically the 16S rRNA gene, has been adopted as a new standard way to identify pathogens in clinical microbiology labs for the past ≈30 years [61]. The interspecies variability characteristic of the gene allows for the identification of unknown pathogens at the species and strain level through the comparison between the obtained sequence and published sequences of known bacterial species and strains [62]. One such system, the MicroSEQ™ 500 16S (Thermo Fisher Scientific), is able to correctly amplify the unknown

pathogen sequence through polymerase chain reaction (PCR) and identify it by comparison with gene libraries in a very short time (1 hour for DNA extraction and ≤ 10 hours for amplification and sequencing). It is especially useful in the identification of rare bacteria [63]. Other nucleic acid-based alternatives such as real-time PCR and fluorescence *in situ* hybridization (FISH) have also drastically reduced the time-to-results, and improved laboratory workflow and patient prognoses [60].

Besides genetic methods, proteomics-based methods were also developed. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is a trending method of ID that is already used in most European microbiology labs [53]. In MALDI-TOF MS, a saturated solution of a low-mass organic compound—a matrix—is added to the sample on a 96-spot metal plate. The matrix and the bacterial sample cocrystallize after drying. The metal plate is inserted into the mass spectrometer and a laser irradiates the crystal, inducing the ionization of the sample. The matrix is essential for this process, as it provides protons for ionization to occur, allowing the analysis of large biomolecules such as proteins. The mass analyser separates the formed ions according to their size and generates a spectrum of their respective mass-to-charge ratios. The obtained spectrum is then compared to spectra of known pathogens available in databases, and similarities in peaks are used to identify the sample pathogen [60, 64].

The main MALDI-TOF MS systems for ID are the Andromas system (Andromas SAS), Vitek® MS (bioMérieux) and the MALDI Biotyper® (Bruker Daltonics), with the latter being the most used platform in the US [60]. These differ mainly in the sample preparation procedure, the species covered in the spectra database and the identification algorithm used [65]. For example, the Biotyper® system has an approximately 20 second sample preparation time per isolate and time-to-results of 30 minutes for a fully used 96-spot plate (Figure 6) [66]. Results are accompanied by a log score between 0.00 and 3.00 for each identification. As per the manufacturer's specifications, if the sample was obtained from colonies, a ≥ 2.00 score is correlated with reliable identification to the species level (represented by the colour green), while a score of 1.70-1.99 is reliable only to the genus level (represented by a yellow colour). Score values < 1.70 should be disregarded as unreliable (represented by the colour red) [67].

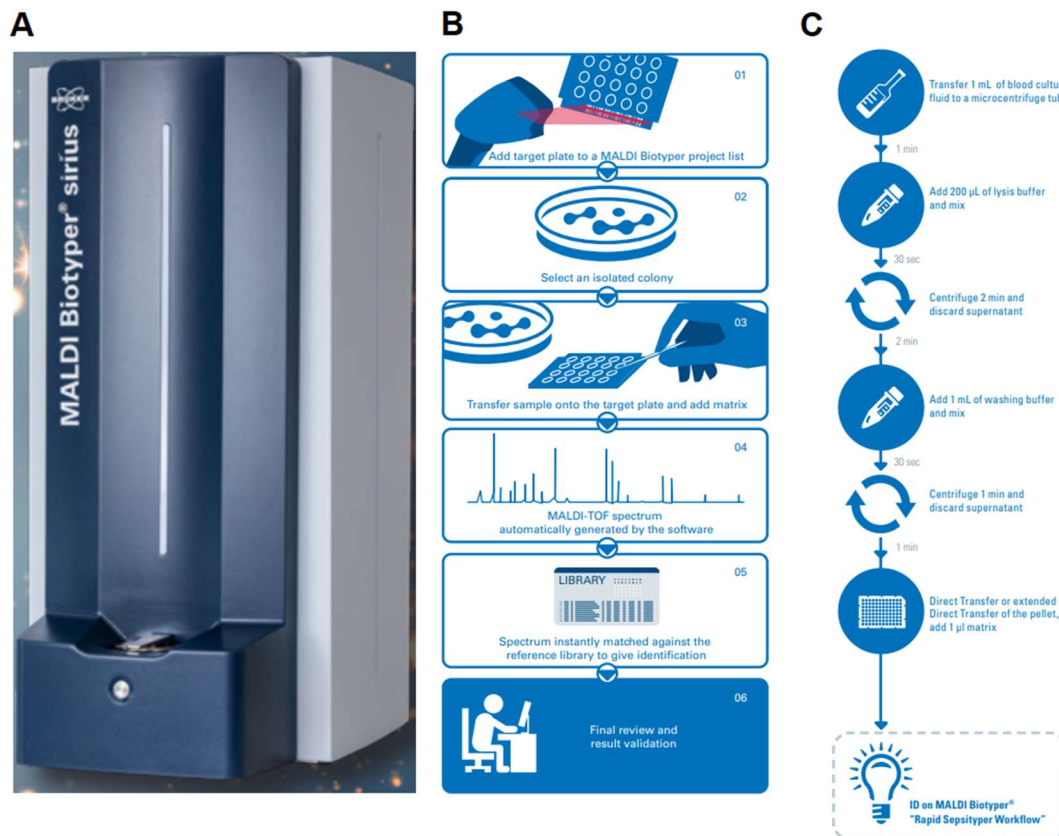


Figure 6: Bruker Diagnostics MALDI Biotyper system. A – Representation of the Biotyper sirius instrument. B – Rapid ID from bacterial colonies. C – Rapid ID from positive blood cultures using the MBT Sepsityper kit. Withdrawn from the MALDI biotyper and Sepsityper kit product brochures [66, 68].

MALDI-TOF MS samples are usually obtained from isolated colonies growing on agar (Figure 6B). This, however, is not optimal for BSI/sepsis diagnosis, as it requires blood culture inoculation in agar medium and subsequent overnight incubation in order to obtain colonies of the unidentified pathogen. As such, several protocols that circumvent the incubation step were developed to reduce the time-to-results. Several rapid identification protocols performed directly from positive blood cultures have been developed, consisting generally in the treatment of a blood culture suspension with a detergent agent, followed by centrifugation and washing steps, enabling a very fast time-to-results of only 20 to 40 minutes. The MBT Sepsityper® kit (Bruker Daltonics) is a commercially available example of a direct identification method that enables ID between 15 and 20 minutes after positive flagging of blood cultures (Figure 6C) [69]. However, as poor implementation of direct methods can lead to suboptimal batch processing of samples, the most used rapid methods involve short subculturing of 4 to 6 hours of positive blood culture aliquots before proceeding with MALDI-TOF MS ID [53, 64].

2.1.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) is the final step before obtaining the susceptibility report, which is the ultimate goal in BSI/sepsis diagnosis, as it allows the shift from empirical to directed treatment.

There are several different AST performance methods. There are standardized methods which are considered references, as they were used for many decades and are still in use today. However, as these are growth-dependent, involve long incubations, and require heavy manual work, automated systems were developed, which are currently in use in many clinical microbiology laboratories. Additionally, the need for further reducing the time-to-results has led to the emergence of several novel technologies that are considerably faster and represent a growing market niche. Several of these methods were discussed onwards in greater detail.

2.1.3.1. Reference methods

Clinical microbiology labs utilize well-established and standardized methods for AST, for pathogens which are known to present clinically relevant resistance. These standardized methods are usually referred to as reference methods, and its procedures and protocols are established by standards-setting organizations, such as the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the Clinical and Laboratory Standards Institute (CLSI) or the International Organization for Standardization (ISO). CLSI admits broth microdilution and macrodilution, agar dilution and disk diffusion as generic reference methods, while EUCAST admits broth microdilution and disk diffusion only. The global standard is considered to be broth microdilution according to ISO [70, 71].

Generally, reference methods can be divided in two categories. Dilution methods consist in the incubation of a bacterial isolate in several serial dilutions of a solution containing antimicrobial agent and subsequent registering of the lowest concentration that visibly inhibits bacterial growth—the minimum inhibitory concentration (MIC). Diffusion methods consist in the incubation of bacterial isolates in agar containing an antimicrobial agent which diffuses through it, and the subsequent measurement of the diameter of an observable halo of inhibition of bacterial growth. Obtained MIC values and halo diameters are compared to breakpoint tables established by the aforementioned standards-setting organizations, which provide interpretive criteria for the determination of susceptibility or resistance of a bacterial species to a certain antibiotic, under the form of breakpoints. This enables the bestowment of categories regarding the susceptibility of the isolate to the assessed antimicrobial, which can be correlated with clinical success of therapy: “Susceptible” (S), “Resistant” (R), or “Intermediate” (I) [71].

The concrete definitions of susceptibility categories present small variations from source to source. According to CLSI, susceptibility is the inhibition of isolate growth by usually achievable antimicrobial concentrations when the recommended dosage is used for that specific infection site. Resistance is defined by lack of growth inhibition under the same circumstances and/or the determination of MIC values or halo diameters that fall into the range associated with likelihood of the presence of antimicrobial resistance mechanisms, and clinical efficacy against the isolate has been shown unreliable in treatment studies. The “Intermediate” category can serve as a buffer zone to avoid interpretative errors; it includes isolates that present a lower response rate than susceptible ones to usually achievable antimicrobial concentrations, and as such can suggest that an increase in dosage can improve clinical efficacy [72]. To reflect this aspect, EUCAST has redefined the “Intermediate” category as “Susceptible, increased exposure”, and CLSI has added a “Susceptible, dose-dependent” (SDD) category for specific antimicrobial-species combinations [73, 74].

2.1.3.1.1. Dilution methods

Dilution methods are based in the incubation of a bacterial sample in serial two-fold dilutions of antimicrobial agent mixed with growth medium. The range of the assessed dilutions should include the established breakpoints specific for the assessed bacteria species and antimicrobial agent. Dilution methods produce MIC values which are compared to established breakpoint concentrations, in order to determine the susceptibility category of the isolate to the assessed antimicrobial agent.

Dilution methods can be performed using with either broth or agar medium. Agar dilution is usually performed in Mueller-Hinton agar, which should be used to prepare several aliquots according to the manufacturer’s instructions, to which the appropriate volume of antimicrobial agent is added to obtain the several serial dilutions. The agar solutions are then poured in plates, allowed to solidify, and can then be stored until needed. After overnight growth in non-selective agar medium, colonies are suspended in saline solution or broth to a turbidity equivalent of 0.5 in the McFarland scale (approximately 1.5×10^8 Colony Forming Units (CFU)/mL). This suspension is further diluted 1:10 (1.5×10^7 CFU/mL), and 1 to 2 μ L are inoculated into a spot in the agar plates, reaching a final inoculum of approximately 10^4 CFU per spot. The plates are then incubated at $35^\circ\text{C} \pm 2^\circ\text{C}$ for 16 to 20 hours, after which the results are read, and growth or lack thereof is determined by comparison with a growth control. This method allows for the simultaneous testing of many isolates through the incubation of several spots per plate,

however, it is very labour intensive and the time-to-results is very slow, as it requires at least two overnight incubations [75].

Broth dilution methods are similar to agar dilution but performed with liquid growth medium instead, usually Cation-adjusted Mueller-Hinton Broth (CAMHB). The broth macrodilution method consists in the preparation of serial two-fold dilutions of antimicrobial agent mixed with broth in test tubes, usually with a volume of 1 mL of solution per tube, each prepared with a concentration twice the desired final value. After overnight growth, colonies are suspended in broth or saline solution, adjusted to 0.5 McFarland standard, and further diluted 1:100 (to approximately 10^6 CFU/mL). 1 mL of the bacterial suspension is added per tube to reach the final volume of 2 mL, effectively halving the final antimicrobial concentration to the desired value, as well as enabling the ideal bacterial concentration of approximately 5×10^5 CFU/mL, which is desirable for broth dilution methods. The tubes are then incubated at $35^\circ\text{C} \pm 2^\circ\text{C}$ for 16 to 20 hours, after which the results are read, and growth or lack thereof is determined by comparison with a growth control. This method is well-standardized, but due to the manual preparation of the serial dilutions, the precision of the method is considered to be ± 1 two-fold dilution [75, 76].

Eventually, the broth macrodilution procedure was miniaturized into the broth microdilution method, for convention and improvement of workflow. The principles of the procedure remain similar, but the test is performed at a smaller scale, in 96-well microdilution trays instead of test tubes, and with much smaller volumes. Each microdilution tray can allow for the testing of ten serial dilutions for one antimicrobial, with two additional wells reserved for the growth control and the negative control. A maximum of eight isolates can be assessed.

For protocols that use a maximum volume of 100 μL per well, serial dilutions of antimicrobial agent are produced similarly to the macrodilution procedure (with twice the concentration of the desired value) and 50 μL of each are pipetted into the wells. A 0.5 McFarland suspension of colonies grown overnight is prepared, further diluted 1:100 and 50 μL are pipetted into each well, obtaining the desired antimicrobial concentration and the ideal bacterial concentration of 5×10^5 CFU/mL. After incubation at $35^\circ\text{C} \pm 2^\circ\text{C}$ for 16 to 20 hours, the results are read in comparison with the growth controls. The economization of reagents and space due to the miniaturization of the procedure are conveniences that the macrodilution procedure does not possess. These conveniences contributed to the widespread use of broth microdilution, which is now considered the international reference method for AST [75, 76].

2.1.3.1.2. Diffusion methods

An alternative to dilution procedures are diffusion methods. These rely in the capability of antimicrobial agents to diffuse through agar, generating a gradient of concentration along its surface.

The disk-diffusion method consists in the inoculation of a standardized bacterial suspension evenly in the surface of agar culture medium with a cotton swab, followed by the placement of preprepared commercially acquired filter paper disks impregnated with a certain antimicrobial concentration. The plates are then incubated at $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ from 16 to 18 hours for most non-fastidious bacterial isolates. After incubation, the results are read (Figure 7). Bacterial growth is visible to the naked eye, and areas where inhibition occurs are easily identified as a halo surrounding the antimicrobial disk, that forms due to the logarithmical decrease in antimicrobial concentration as the distance from the disk increases. The diameter of the halo of inhibition is measured and then compared with established diameter breakpoints in order to provide a susceptibility category to the assessed isolate [75].

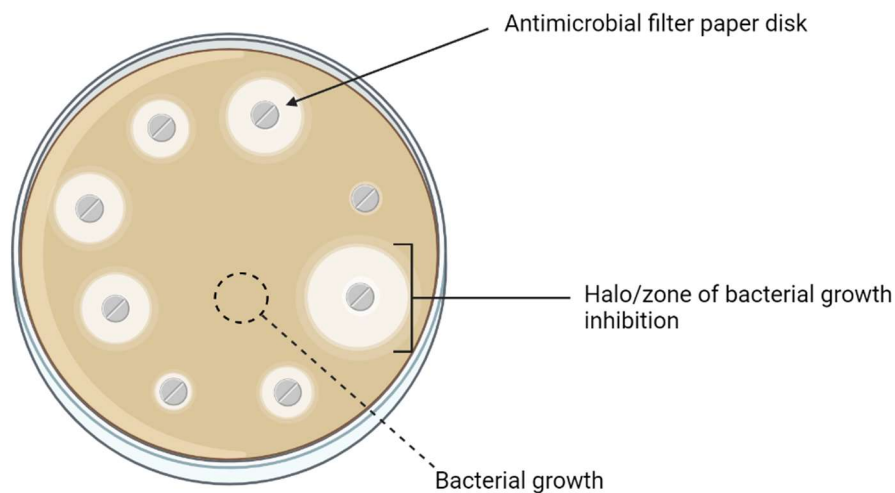


Figure 7: Illustration of a possible resulting Petri dish after a disk-diffusion antimicrobial susceptibility test. Illustration designed on BioRender [52].

As the test results are inhibition zone diameters, a quantitative MIC value is not obtained, contrary to dilution procedures. However, the test is simple to perform, has low associated costs, the results are easy to interpret, and it allows for the testing of a great number of isolates and antimicrobials [77].

2.1.3.2. Automated AST systems

Commercialized systems for AST have been introduced in the 1980s and have been used extensively in most clinical laboratories since the 1990s. The commercialization of such systems is regulated in order to assure the quality of their performance. In the United States, the commercialization of alternative AST methods is dependent on the granting of clearance by the Food and Drug Administration (FDA). In Europe, it is necessary to comply with European Union legislation through the obtainment of a CE marking for *in vitro* medical devices (IVD). Before being commercialized, these tests are subject to several validation studies to assess their performance versus the established reference methods. Several indicators such as essential agreement, categorical agreement, and minor, major and very major errors are used to evaluate the performance of the assessed methods [78].

Several manual and semi-automatic diffusion and broth dilution systems are available, but the discussion in this section was focused on automatic broth microdilution systems, which do not require additional manual work after the placement of the test in the instrument.

There are three main automated broth microdilution systems available in Europe, the VITEK® 2 (bioMérieux), MicroScan WalkAway *plus* (Beckman-Coulter) and BD Phoenix (Beckton-Dickinson) (Figure 8). The hands-on time and manual effort needed for these systems are very low, with most of the AST process being completely automated.



Figure 8: Pictures of automated AST instruments. A – VITEK 2; B – MicroScan WalkAway 96 plus; C – BD Phoenix M50. Obtained from the respective manufacturer's websites.

These are extensively used in larger clinical microbiology laboratories throughout the world and generally consist in single use AST panels containing wells with several concentrations of antimicrobials in which the isolate samples are inoculated, that are inserted into an instrument which incubates the panels and is capable of reading and providing the results of the test [79].

The sample throughput of automated systems is generally very high, reaching up to 120 panels per instrument in the case of the VITEK 2 system (Table 1). Bacterial growth is usually detected by either turbidimetric or colorimetric readings, depending on the system. Besides AST, these systems also allow for the use of ID or mixed AST/ID panels, permitting the identification of the pathogen alongside the performance of the AST in the instrument, without the need to perform an additional ID procedure such as MALDI-TOF MS.

Despite the increase in conveniency and improvement of laboratory workflow associated with the usage of these systems, the time-to-results is usually very variable and inherently slow due to its dependence on the growth of the bacterial isolate.

Table 1: Comparison of automatic broth microdilution commercial systems. Information obtained from the manufacturer's websites, time-to-results obtained from (Behera, B. et al., 2019) [80].

System	Detection technology	Throughput (number of panels per instrument)	Time-to-results (hours)
VITEK 2	Turbidimetric	60 (VITEK 2)	4 - 16
		120 (VITEK 2 XL)	
		15, 30 or 60 (VITEK 2 COMPACT)	
MicroScan WalkAway <i>plus</i>	Turbidimetric	40 (WalkAway 40)	4.5 - 18
		96 (WalkAway 96)	
BD Phoenix	Colorimetric and turbidimetric	99	6 - 16

2.1.4. Novel AST methods – rapid AST

The importance of reducing the time until AST results are obtained relies on the possibility of performing antimicrobial de-escalation sooner. Ideally, the adjustment to treatment with narrower-spectrum antimicrobials should be done as early as the second dose of administration, which in most cases is between 6h and 8h of the first dose. Despite the improvement in automation, workflow, throughput, and time-to-results seen with the first generation of automated AST, this timeframe was difficult to be achieved. As such, there was a need for even faster AST methods, that provided results capable of allowing a faster transition to targeted therapy [81].

This need has sparked an interest in the development of novel rapid AST (rAST) technologies, which led to the appearance of several companies presenting their own solutions to this problem, each with its own advantages and downsides. Some of these were briefly reviewed onwards. This information was gathered for a review of emerging

and established rAST technologies on the market, performed in the scope of this internship at FASTinov.

2.1.4.1. Accelerate Pheno® (Accelerate Diagnostics)

Accelerate Diagnostics is a US company founded in 1987 and re-established in 2012. Its flagship product, the Pheno® system, is a rAST method with optional bacterial ID. It is CE-IVD marked and FDA-cleared, and was one of the first novel rAST methods to be introduced in the market.

Sample preparation is hands-free, performed directly from positive blood cultures, and fully automated. After its loading in the instrument, the sample is cleaned of unwanted debris through gel electrofiltration, automatically diluted to the required concentration, and bacteria cells are positioned and fixed to a surface to permit analysis.

The system is able to perform identification of common BSI-causing pathogens through automated FISH. AST is performed through morphokinetic cellular analysis, which consists in the monitoring of cell growth by automated microscopy after antibiotic exposure. A recent evaluation of the performance of the system claims a time-to-results of 7.09 hours, which consists in a reduction of 41.86 hours when compared to standard of care [82]. The overall reduction in the time until definitive therapy associated with the implementation of the Pheno® system appears to be of 16 hours. AST can be performed both for gram-positive and gram-negative bacteria, and a total of 78 bacteria-drug combinations were assessed [83].

2.1.4.2. QuickMIC® (Gradientech AB)

Gradientech AB is a biotechnology company based in Uppsala, Sweden, which specializes in the development of microfluidics-based products. Their rAST test, QuickMIC® relies on this premise to obtain phenotypic MIC results directly from positive blood cultures. Although CE-IVD marked since May 2022 (only the gram-negative cassette), it is not yet for available for sale [84].

Two cassette types are provided, one for gram-positive and one for gram-negative bacteria, both of which come pre-filled with twelve antibiotics. The workflow of the assay begins with the loading of the bacterial sample mixed with agarose into the culture chamber of the cassette, which is then allowed to solidify. Two channels flanking the culture chamber are loaded with culture medium broth with the aid of a syringe, with one

of these channels coming pre-filled with antibiotic. This establishes a continuous and linear gradient of antibiotic concentration in the culture chamber through diffusion from the antibiotic-containing channel to the medium-containing channel [85]. The cassette is then loaded in the QuickMIC® Instrument, which runs the assay and monitors colony growth in real-time throughout the culture chamber, determining the minimum concentration for which no growth is observed. The QuickMIC® Analyst software automatically reads the assay and provides clinically actionable results, delivering MIC values with a time-to-results of between 2 and 4 hours [86]. Due to the shorter time-to-results even in comparison with other rAST, the company refers to their AST as being in the ultra-rapid category.

The test covers a total of 117 bacteria-drug combinations (83 in the gram-positive panel and 34 in the gram-negative panel). The instrument allows for the analysis of one patient sample against a panel of 12 antibiotics per run (one cassette). However, the system is somewhat scalable, as up to 12 instruments can be stacked and ran simultaneously. The company claims the linear gradient of concentrations allows for better resolution, precision, and accuracy, as the assessed range is considerably narrower than for example in broth microdilution, which relies in two-fold serial concentrations, and thus can be focused on clinical breakpoints.

There are, however, some acknowledged disadvantages. Despite the advantages associated with the linear concentration gradient, the MIC value of certain strains can potentially be outside its very narrow concentration range. This is admitted as the main disadvantage of the product. Another disadvantage is the fact that the test is growth-dependant, which indicates that time-to-results is inevitably affected by the growth speed of the assessed strain. The throughput is also not ideal, as only one patient sample is analysed per run, however, a 2020 publication by the company mentions the development of a multiplex chip that allows for a total of eight patient samples per run [87].

2.1.4.3. ASTar (Q-Linea)

Q-linea is a biotechnology company founded in 2008, in Uppsala, Sweden, focusing on the diagnostic of infectious diseases. Their flagship product, ASTar, was developed as an AST-only assay capable of matching the speed of emerging fast ID methods. The ASTar gram-negative kit is CE-IVD marked since 2020 and the ASTar instrument recently obtained CE-mark for IVDR (*In vitro* Diagnosis Regulation) in 2021 [88]. The product has already launched in the European market in partnership with Thermo Fisher

Scientific, and one Swedish regional hospital has already signed an evaluation contract [89]. Although not cleared for sale in the US-market, an application for FDA-clearance was recently submitted [90].

The ASTar assay is performed directly from positive blood cultures and requires only 2 minutes of hands-on time. 1 mL of positive blood culture is transferred to the sample preparation cartridge, which contains all reagents and disposables needed for the performance of the assay. Then, the AST Disc is loaded in the ASTar Instrument and the run is ready to start [91].

The technology is based in broth microdilution optimized for a shorter time-to-results, enabling the delivery of true MIC results within 6 hours. The AST disc contains more than 330 culture chambers prefilled with 6 to 14 two-fold concentrations of antibiotics, control chambers, and chambers used to determine the concentration of the loaded blood sample [92]. At first instance, 6 patient samples can be loaded, with the potential to add 6 more 50 minutes past the start of the run, for a total throughput of 12 samples. Although the antimicrobial coverage of the assay is wide, it is currently only usable for gram-negative bacteria.

2.1.4.4. Nanomotion-AST (Resistell™)

Resistell™ is a Swiss startup company founded in 2017, which is developing its own rAST. As of 2022, the test is not available for sale, nor is CE-IVD marked. The technology of this assay is based in nanomotion detection. It is not dependent on growth of the assessed bacteria, which is an advantage in the reduction of time-to-results.

The assay is performed directly from positive blood cultures. However, a preparation protocol is needed in order to obtain bacterial pellets, which takes less than 1 hour. The bacterial isolate is then attached to mechanical cantilevers, which oscillate if the bacteria are metabolically active. After exposure to an antibiotic, oscillations are recorded through the means of a laser and a detector, and a reduction in signal is correlated to susceptibility to the concentration in question. This enables the determination of MIC values in between 1 and 2 hours, in a growth-independent manner [93].

As this rAST appears to be still in development, the details of the procedure, workflow, throughput, and potential instrument are not yet available. A clinical pilot evaluation of the assay in partnership with the Lausanne University Hospital was completed in December 2021, and the AST presented results with 100% accuracy in comparison with disk-diffusion method and the VITEK 2 system. However, only one gram-negative

bacteria-drug combination was tested. A 4 hour time-to-results was observed in this evaluation, which was slightly longer than the one advertised in the website [94].

2.1.4.5. Specific Reveal® rAST system (Specific Diagnostics)

Specific Diagnostics is a US-based company, located in San Jose, California. Their rAST technology, Specific Reveal®, is based in the detection of volatile compounds emitted by bacteria during growth. It is CE-marked and available for use outside of the US but not yet FDA-cleared [95]. European distribution of the product is done in partnership with bioMérieux.

Sample preparation takes 3 minutes or less, involving only the dilution of the positive blood culture in a 96-well panel, which comes pre-filled with several concentrations of antibiotics, and the attachment and sealing of a sensor sheet to the aforementioned panel. Panels are then ready to be loaded in the Specific Reveal® instrument, which has two drawers for plate insertion, accommodating two plates per drawer. The sensor panel has sensors in each well that change colour when exposed to volatile compounds emitted by growing bacteria, and this change of colour is monitored by scan every 10 minutes. The monitorization of the percentage of colour change per well is then used to determine the MIC value in an average of 5.5 hours, and as low as 3.5 hours [96].

The instrument has two drawers, each of these accommodating two panels. This allows for a total throughput of 4 patient samples per instrument. Similarly to the QuickMIC® system, instruments can also be treated as modules and ran simultaneously, which permits the scaling of the assay up to a maximum of 6 modules, or 24 patient samples per shift [96]. The test was subject to a total of eleven clinical validations across England and France, yielding an overall accuracy of approximately 97.6%, and it is already used in two clinical microbiology laboratories in France [97]. However, it is usable for gram-negative bacteria only at the moment.

2.1.4.6. Next-Generation Phenotyping™ (Selux DX)

Selux is a Boston, Massachusetts-based company developing a novel, phenotypic rAST system, Next-Generation Phenotyping™, which is based in cell surface antibody binding to determine morphological changes.

Prior to cell death, exposure to antibiotics causes severe morphological changes in bacteria cells. In the case of β -lactam antibiotics, which account for 70% of prescribed

antibiotics in the US, temporary elongation or swelling can occur when bacteria are exposed to concentrations above the MIC, and septation does not occur. The system exploits this phenomenon by incubating bacteria cells with a surface-binding antibody after antibiotic exposure. After a single, endpoint signal measurement, a reduction in signal intensity when compared to a positive growth control is correlated to susceptibility, as elongated bacteria present less total surface area than resistant, fully dividing bacteria [98].

The system provides results in up to 5 hours in 99% of cases [99]. As it is a single endpoint measurement assay, it does not need periodic monitoring of cell growth. As it presents compatibility with the use of microplates and microplate readers, the assay is expected to allow for high throughputs at a low cost, allowing the assessment of several antibiotics in parallel. However, the assay is dependent on the growth rates of the tested strains, and MIC determination will inevitably take longer in bacteria with slower doubling rates [98].

2.1.4.7. Astrego Diagnostics rAST system

Astrego Diagnostics AB is an *in vitro* diagnostic company based in Uppsala, Sweden. The company is currently developing a rAST system which is CE-IVD marked for usage with urine samples for urinary tract infections only as of 2022 [100]. However, the technology is expected to permit the usage of sample types other than urine, such as isolated colonies or blood cultures. As such, it is expected to be useful for several conditions other than urinary tract infections, such as respiratory infections, tuberculosis, and sepsis [101].

The system is based on microfluidics and single-cell imaging. The sample is loaded in an indication specific cartridge, a microfluidic chip, which is then loaded in the instrument. Bacteria are caught in single cell-sized microchannels in the chip, with one fraction of the population being exposed to an antibiotic. Bacterium exposed to antibiotic are monitored for growth, which is then compared to the reference population unexposed to the antibiotic. Inhibition of growth is checked to determine susceptibility [101].

Results for sepsis are expected to be delivered in 5 to 60 minutes after gram-staining. The process of averaging of growth rate of several hundred bacteria cells permits a very fast detection of changes in growth provoked by antibiotic exposure, which allows the system to circumvent the increase in time-to-results that affects other growth-dependent rAST [102]. Details regarding throughput and antibiotic coverage are still not available.

2.1.4.8. Flow cytometry and AST

2.1.4.8.1. Brief introduction to flow cytometry

Flow cytometry is a technology that enables multiparametric analysis of cells or other particles.

A sample is injected on a flow cytometer, the instrument responsible for the procedure. The injected liquid sample enters through a fluidics system in the flow cell and travels in the middle of pressurized sheath fluid, which generates laminar flow (Figure 9A). This process is called hydrodynamic focusing, as it narrows the sample flow using fluid dynamics, leading cells present in the sample to flow one at a time in a single-file line through the interrogation point, enabling cell counting and individual cell analysis [103-105].

Cells are analysed for visible light scatter as they pass through lasers in the interrogation point (Figure 9B). The lasers act as a source of light, which is refracted as it travels through the cells. The light that is refracted forward to the direction of the laser—forward scatter—is proportional to cell size and can be captured by a detector directly facing the laser. The light that refracts in other directions—side scatter—is captured by a detector perpendicular to the laser direction and is proportional to the structural complexity and granularity of the cell. The detected light intensity is converted into a digital signal by the electronics system and the obtained data can then be analysed by a computer [103, 106]. Forward and side scatter can be plotted, differentiating cells by size and structural complexity in a heterogenous sample, such as blood for example.

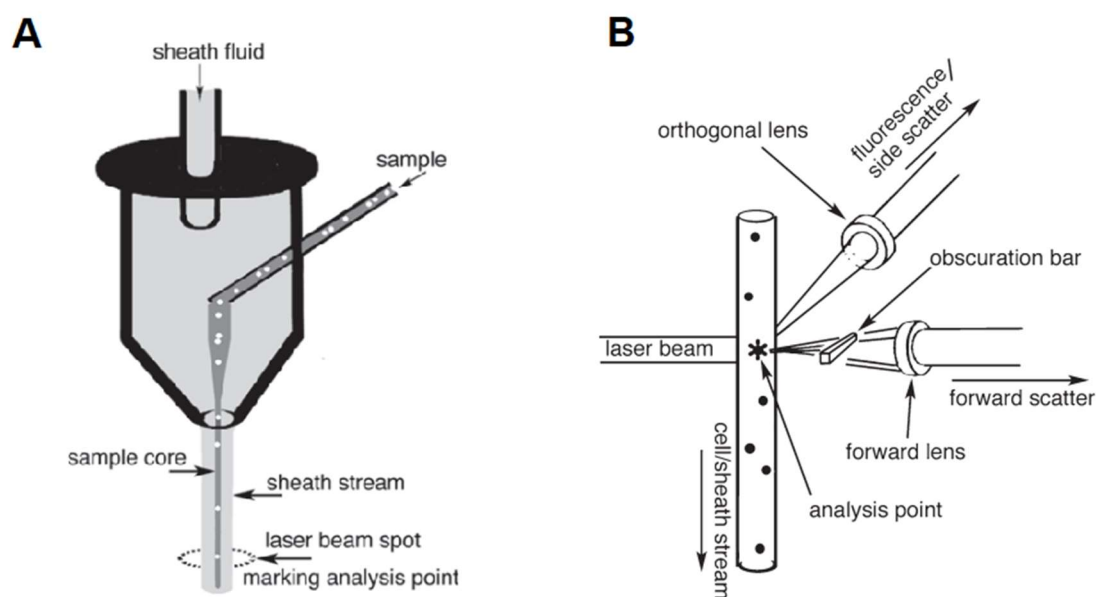


Figure 9: Illustrations of the components and function of the flow cytometer. A – Representation of hydrodynamic focusing, with a close-up on the flow cell. B – Representation of the interrogation point. Withdrawn from (Given, 2011) [107].

Besides forward and side scatter, the detection of fluorescence is also possible with flow cytometry. Specific wavelength lasers can excite fluorochromes present in cells, which then emit light at a longer wavelength (fluorescence). The emitted light passes through wavelength filters and is delivered to several fluorescence detectors, which detect the emitted fluorescence by wavelength ranges (colours). This is useful for the detection of target molecules present in cells, through the labelling with fluorochrome-conjugated molecules (antibodies) that have affinity for the target. A graph can then be plotted to identify cells that present the target, as its presence is proportional to the detected fluorescence intensity [108].

The analysis of multiple parameters through fluorescence-labelling confers potential to this technology to be useful in several different clinically related fields, and as such, flow cytometry is commonplace in immunology and haematology laboratories [109, 110].

2.1.4.8.2. Flow cytometry as an AST method

Since bacteria are single-celled organisms, flow cytometry can be applied to microbiology. Efforts for this began in the 1970s and 1980s, with the application of flow cytometry-based methods for rapid bacterial ID and AST. However, since microbiology in general is not a field highly associated with innovative high technology, which is easily observed by its reliance on mostly cheap and classical growth-dependent methods, there was a lack of interest in newer and more expensive technology at this time. As such, the design of flow cytometer instruments able to analyse smaller particles such as bacterial cells, which are considerably smaller than mammalian cells, was not considered to be relevant, which in turn appears to have contributed to the initial disinterest in flow cytometry for clinical microbiology [109]. The interest has increased over time though, especially due to its potential as an AST method, through the usage of fluorescent molecules that function as markers of cell viability, allowing the assessment of the susceptibility or resistance of a bacterial isolate to the effects of an antimicrobial agent.

There are essentially two groups of fluorescent molecules described in literature to be used for the assessment of viability after antimicrobial exposure: DNA- or RNA-labelling dyes and metabolic/protein-binding dyes. Mithramycin and ethidium bromide are DNA-binding dyes which can assess cell viability after exposure to β -lactam antimicrobials, which induce an increase in nucleic acid contents in cells undergoing filamentation, i.e., cells that elongate but cannot divide. Another example of a DNA-binding probe is propidium iodide (PI), which can enter bacterial cells presenting loss of membrane integrity after antimicrobial exposure and bind to DNA [111]. Acridine orange is a dye

that is able to simultaneously bind to and stain DNA and RNA in a differential manner, as the emitted fluorescence is of different wavelengths (530 nm for DNA staining and 640 nm for RNA staining). As such, it can be used as a probe to determine loss of viability due to antimicrobial susceptibility, as cells that are unable to divide present decreased RNA quantity, while viable, actively dividing cells present more RNA [112].

An example of the group encompassing metabolic or protein-binding dyes is the anionic hydrophilic dye *Bis*-(1,3-dibutyl-barbituric acid) trimethine oxonol (DiBAC₄ (3)), which binds to membranes and intracellular proteins of cells that suffer from depolarization of the membrane as a consequence of antimicrobial exposure [113]. On the other hand, Rhodamine 123 is a cationic lipophilic dye that accumulates in cells with an inside negative transmembrane potential. These two dyes can be used in conjunction for the determination of membrane potential and consequently cell viability as they present opposite effects, with cells presenting depolarized membranes accumulating DiBAC₄ (3) and having a decreased uptake of Rhodamine 123, and vice-versa [114].

2.1.4.8.3. FASTinov flow cytometry AST

FASTinov S.A. is a company based in Porto, Portugal, that developed a product based in multiparametric fluorescence flow cytometry for the performance of Flow Cytometry Antimicrobial Susceptibility Testing (FAST) directly from positive blood cultures. It relies on a proprietary combination of fluorochromes that act as early markers of microbial cell lesions and metabolic changes associated with antimicrobial exposure, which are able to be identified in a flow cytometer [115, 116].

FASTinov provides three different CE-IVD marked kits for FAST. Each kit includes a pre-prepared 96-well AST panel containing dehydrated antimicrobial agents and fluorochromes, besides reagents and consumables. After a 5-to-10-minute sample preparation procedure, which includes washings and centrifugations to clean the sample and obtain a bacterial pellet, the resulting suspension can be inoculated into the panel. The panel is incubated at 37°C for 1 hour with agitation, to expose the isolate to the antimicrobial agent and to resuspend the dehydrated reagents.

After the incubation, the panel is inserted in a flow cytometer which reads the fluorescence levels in each well, followed by the delivery of the AST report with the susceptibility categories of the assessed isolate by the proprietary bioFAST software. The time from the initiation of sample preparation to the generation of the AST report is one of the fastest available: 2 hours.

A total of three AST kits are available (Figure 10). Two of these are for gram-positive or gram-negative bacteria, FASTgrampos and FASTgramneg kits, respectively. The FASTgrampos kit includes FAST for 11 antibiotics for use with *Staphylococcus* and *Enterococcus* spp., and several concentrations of vancomycin for MIC determination for *S. aureus*. The FASTgramneg kit includes 13 antibiotics for *Enterobacterales* spp., *Pseudomonas* spp. and *Acinetobacter* spp., as well as detection of common mechanisms of resistance. A third kit, FASTcolistin MIC, includes several dilutions of colistin for MIC testing in gram-negative bacteria. This incurs in a total of 236 validated bacteria-drug combinations.



Figure 10: Pictures of the currently available FASTInov AST panels. From left to right: FASTgrampos, FASTgramneg and FASTcolistin MIC.

Although FASTInov AST is currently performed from positive blood cultures, the technology has potential to be used with other sample types. The kit was also validated using bacterial strains isolated from companion animals, potentially indicating its usefulness for veterinary purposes [117].

3. Objectives

The objectives in the scope of this internship at FASTinov S.A. were:

- The familiarization and practice with reference AST methods and its comparison with FAST.
- The performance of a comparison study between two procedures of direct ID by MALDI-TOF MS that can be integrated in the workflow of the AST method provided by FASTinov.
- The assistance in the launch of FASTinov's products in the market, through the preparation of promotional material and planning for the attendance of scientific congresses.

Materials and Methods

Comparison between FAST and reference AST methods

The performance of the FASTgrampos and FASTgramneg kits was assessed in comparison to reference AST methods (broth microdilution and disk-diffusion tests). FAST and AST were mainly performed with clinical bacterial isolates obtained from FASTinov's strain collection, which were stored in Brain Heart Infusion (BHI) broth with 10% glycerol, at -80°C.

Additional reference strains with different susceptibility phenotypes were obtained from the American Type Culture Collection (ATCC) and Culture Collection University of Gothenburg (CCUG) and were included in the study (Supplementary Table 1).

An overview of the total number of assessed isolates was summarized in Table 2. A total sample size of 311 was considered for this study, of which 180 strains were gram-negative bacteria, and 131 were gram-positive bacteria.

All handling of biological material was performed in a safe laminar flow cabinet.

Table 2: Per species, per group and total number of gram-negative and gram-positive isolates assessed for FAST and reference AST.

Gram-negative bacili		Gram-positive	
<i>Enterobacterales</i>	78	<i>Staphylococcus spp.</i>	66
<i>Escherichia coli</i>	15	<i>S. aureus</i>	35
<i>Klebsiella pneumoniae</i>	41	<i>S. epidermidis</i>	23
<i>Klebsiella oxytoca</i>	-	<i>S. hominis</i>	8
<i>Klebsiella aerogenes</i>	-	<i>Enterococcus spp.</i>	65
<i>Serratia marcescens</i>	3	<i>E. faecalis</i>	44
<i>Providencia rettgeri</i>	1	<i>E. faecium</i>	18
<i>Proteus mirabilis</i>	5	<i>E. gallinarum</i>	2
<i>Enterobacter cloacae</i>	9	<i>E. casseliflavus</i>	1
<i>Enterobacter aerogenes</i>	4	Total	131
Non-fermenters	102		
<i>Pseudomonas aeruginosa</i>	72		
<i>Acinetobacter baumannii</i>	30		
Total	180		

Determination of susceptibility by broth microdilution

1. Broth microdilution plate production

The production of ready-to-use plates for MIC determination by broth dilution was performed as per the CLSI M07-A10 document (Figure 11) [118]. MIC breakpoints for each antibiotic were used to establish the maximum and minimum tested concentrations. All steps in this procedure were performed in a laminar flow cabinet not used for handling of biological material, to prevent any contamination.

A small amount of antimicrobial was weighed in a high precision scale and diluted in 1 mL of its respective solvent, as indicated in the guidelines. This stock antimicrobial solution was diluted in CAMHB in a 15 mL tube, taking into account the weighed mass of antimicrobial and the lot-specific potency indicated in the certificate of analysis, in order to obtain the solution with double the concentration of the desired maximum tested concentration. This solution was the starting point for nine further serial 1:2 dilutions using CAMHB as a solvent, obtaining a total of ten solutions with decreasing concentrations of antimicrobial.

Each of these solutions was poured into a micropipette reservoir and was pipetted into one column of a 96-well microdilution tray with an 8-channel micropipette, pipetting 50 μ L of solution in each well. The two remaining columns were used for the positive and negative control of the procedure. The positive control wells contained only CAMHB, while 50 μ L of the lowest concentration antimicrobial solution were added to the negative control well.

The produced plates were identified and refrigerated if meant to be used in the same day or stored at -80°C for later utilization.

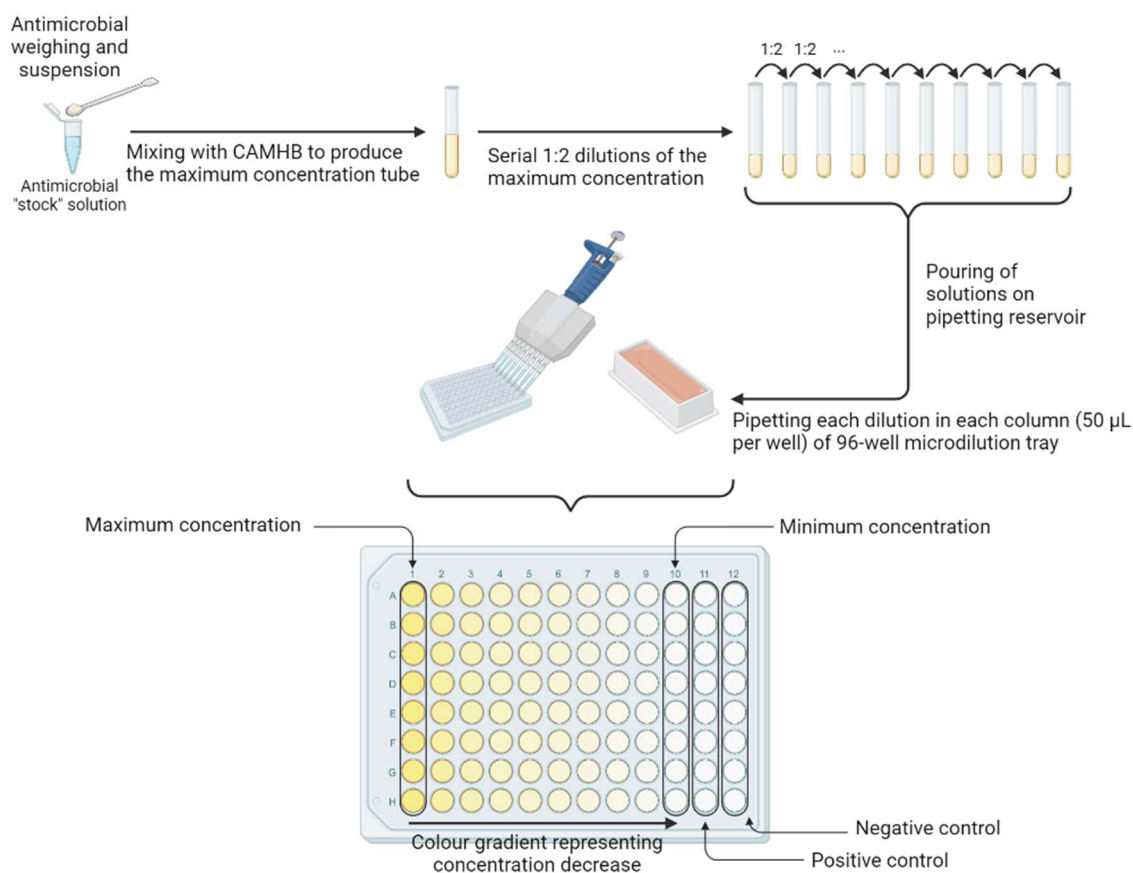


Figure 11: Schematics of the workflow involved in broth microdilution plate production. Illustration designed on BioRender [52].

2. Determination of susceptibility by broth microdilution

Determination of MIC of an antimicrobial by broth microdilution was performed as per the CLSI M07-A10 document (Figure 12). In preparation for this procedure, the frozen bacterial isolates were cultured in MHA and incubated overnight at 37°C. For each isolate, a small amount of biological material was collected using an inoculation loop and transferred to a densitometer tube containing approximately 2 mL of 0.9% saline solution. The turbidity of the resulting suspension was measured using a densitometer and was adjusted for McFarland Standard 0.5 (approximately 1.5×10^8 CFU/mL) by adding more biological material or mixing in more saline solution, and the adjusted suspension was 1:100 diluted. Using a micropipette, 50 µL of this solution were pipetted per well, into each of the ten concentration wells of the broth dilution 96-well plate, as well as in the positive control (final volume for each well: 100 µL). In the negative control well, 50 µL of CAMHB were pipetted instead. The plate was then incubated overnight at 37°C.

After incubation, each well was checked under light for turbidity to identify bacterial growth. The lowest concentration with no observable growth for each isolate was

registered as the MIC. The obtained MIC values were then cross-referenced with MIC breakpoint tables present in EUCAST and CLSI guidelines, and a susceptibility result (S – Susceptible; R – Resistant; I – Intermediate) was granted to each strain for each tested antimicrobial.

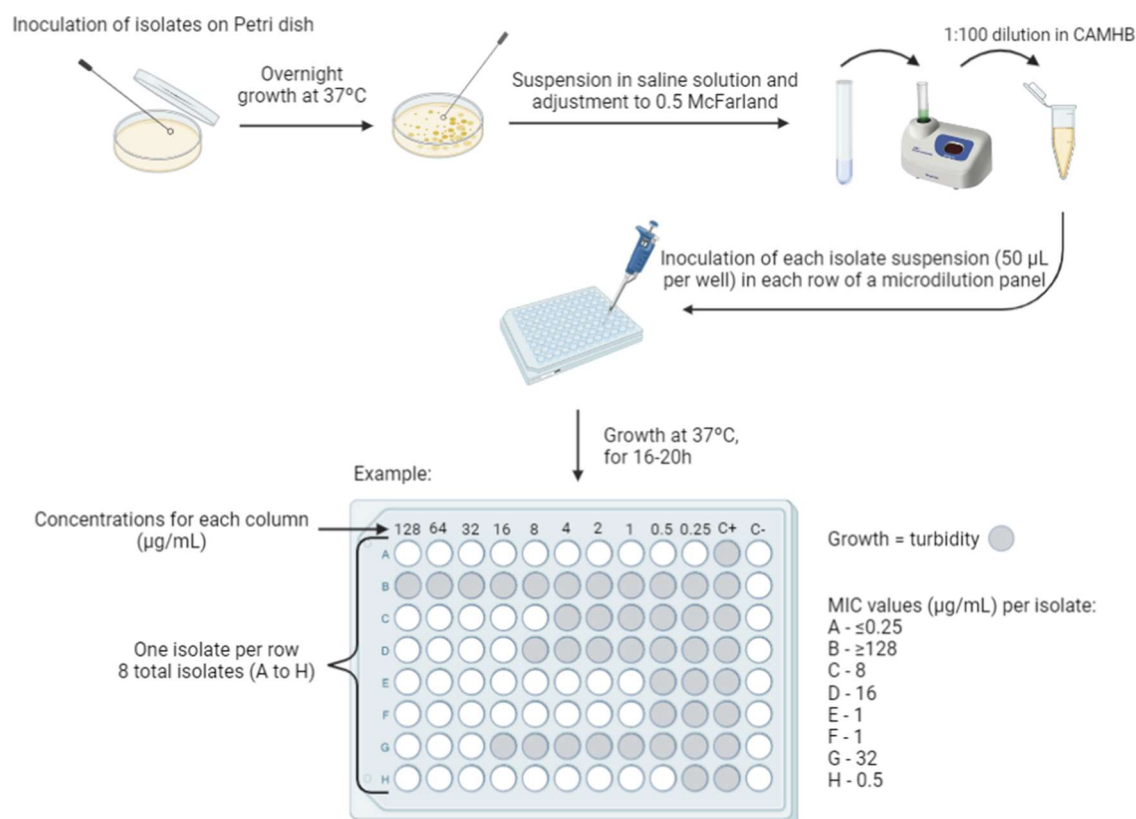


Figure 12: Schematics of the workflow involved in the determination of MIC by broth microdilution, with an example of a resulting microdilution panel after incubation. Illustration designed on BioRender [52].

Determination of susceptibility by disk diffusion

Determination of the susceptibility to an antimicrobial by disk diffusion was performed according to the EUCAST protocol (Figure 13) [119]. The stored frozen isolates were cultured and incubated overnight at 37°C, and a suspension in saline solution was adjusted for McFarland Standard 0.5 as previously explained. Using a cotton swab, this solution was spread throughout the surface of a new Petri dish containing MHA. Using metal tweezers, antibiotic disks were placed onto the medium surface and gently tapped to assure their fixation to the agar medium. Petri dishes were then incubated overnight at 37°C.

After incubation, the halo of inhibition was measured for each antibiotic disk. Halo diameter values present in EUCAST and CLSI tables were used as references to determine the rate of susceptibility to the antibiotic in question of the tested isolate.

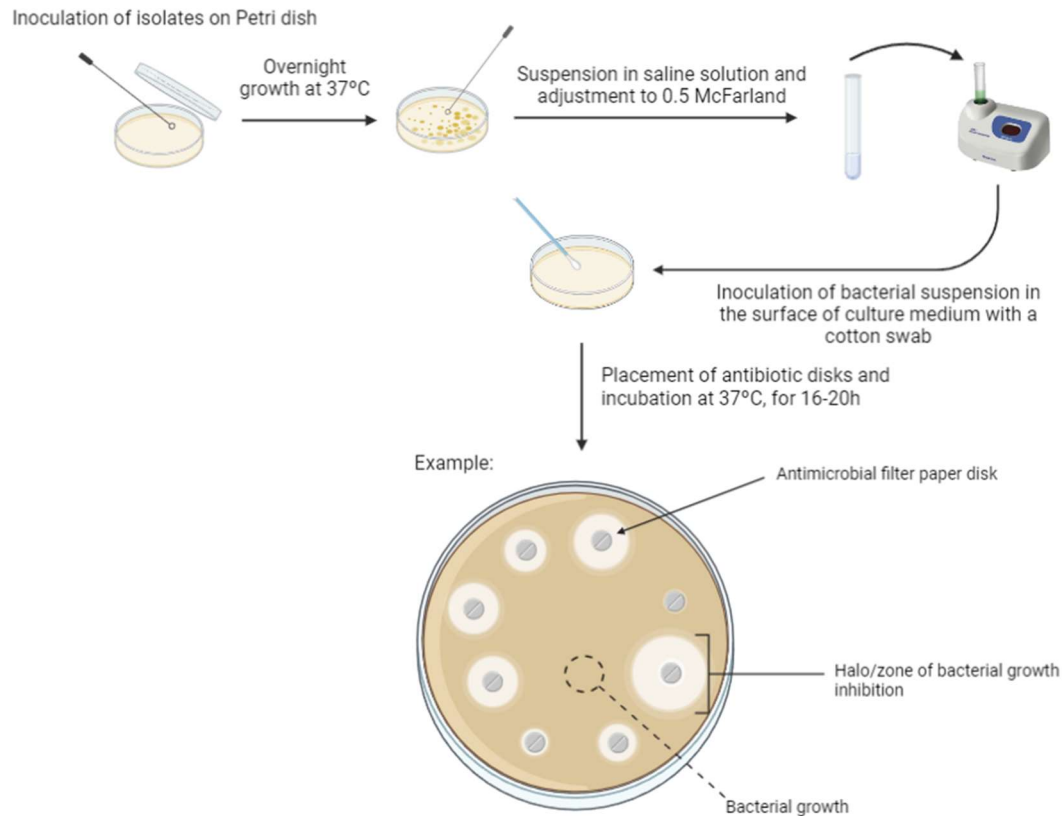


Figure 13: Schematics of the workflow involved in the determination of susceptibility by disk-diffusion testing, with an example of a resulting Petri dish after incubation. Illustration designed on BioRender [52].

Determination of susceptibility by FAST

As FAST is performed directly from blood cultures, both the stored isolates and the reference strains were inoculated in blood culture bottles. Bacteria were cultured and incubated overnight at 37°C, suspended in saline solution and adjusted for McFarland Standard 0.5, and further diluted 1:100 in CAMHB medium as previously explained. Using a 10 mL syringe, between 8 and 10 mL of healthy donor blood obtained from Centro Hospitalar Universitário de São João (CHUSJ) were transferred to an aerobic or anaerobic blood culture bottle. Approximately 0.1 mL of the diluted bacterial suspension were inoculated in the same bottle using a 1 mL syringe. The spiked blood culture bottles were identified with the designation of the inoculated isolate, was well agitated, and incubated overnight in a BACTEC incubator (BD). The incubated blood cultures were flagged positive usually in the following morning, after which FAST was performed.

FAST was performed as per a patented protocol referenced in the instructions for use (IFU) of FASTinov's products. 1 mL of positive blood culture was extracted from the bottle and transferred to a tube with a haemolytic agent and vortexed for 10 seconds. The tube was then centrifuged at 13000 revolutions per minute (RPM) for 1 minute and the supernatant was rejected, after which the pellet was resuspended in 1 mL of sterile 0.9% saline solution by vortexing. The contents from this tube were gently pipetted to a second tube containing a gradient-separating agent, which was then centrifuged on the same conditions as before. The supernatant was rejected, and the obtained bacterial pellet was resuspended in 1 mL of saline solution, which can be stored at 5°C if not inoculated right after.

The resulting solution was adjusted for McFarland Standard 0.5, after which 1 mL was transferred to a tube containing 7 mL of pre-prepared CAMHB medium. If the isolate was identified as *Staphylococcus* spp. or *Pseudomonas* spp., 2 mL of the McFarland adjusted solution were transferred instead.

Using a 12-channel micropipette, 100 µL of the poured solution per well were transferred to the AST panels, and the "up & down" technique was performed to resuspend the dehydrated content in the wells. Depending on the kit, different rows were inoculated:

- FASTgrampos: rows A to C
- FASTgramneg: rows A to E

After the inoculation procedure, the panels were incubated at 37°C, with agitation, for 1 hour, avoiding direct light to protect the correct function of the fluorochromes. After incubating, the panels were inserted into a CytoFlex (Beckman Coulter) flow cytometer and read.

Validation of the performance of two direct methods for MALDI-TOF MS ID

As previously stated, the identification of the pathogen is nowadays mandatory before proceeding with the AST, as breakpoint concentrations are specific to taxonomy.

In order to incorporate a direct method of rapid ID by MALDI-TOF MS in the workflow of FAST, the performance of two different pellet-based methods directly from positive blood cultures was assessed. The performance of both of these methods was determined in comparison to conventional MALDI-TOF MS ID from overnight bacterial colonies, which served as the reference method.

Although direct ID using the previously mentioned MBT Sepsityper kit (Bruker Daltonics) was also performed, it was not considered for the comparison as it was not used for all samples. While this method is simple to perform, it is expensive and as such not widely used.

Sodium dodecyl sulfate ID method

The first method was performed as described in a study at Hospital Universitario Ramón y Cajal, Madrid [43] (Figure 14).

After the completion of the steps in the FAST protocol that require manual intervention, the performance of bacterial isolation for ID can start. For this method, 1 mL of blood culture and 200 μ L of a 5% solution of sodium dodecyl sulfate (SDS) were added to a sterile microcentrifuge tube and mixed on a vortex. The resulting solution was centrifuged at 13000 RPM, for 1 minute. After discarding the supernatant, the resulting pellet was resuspended in 1 mL of sterile distilled water. Two more cycles of vortexing followed by centrifugation, supernatant disposal and resuspension in water were performed, after which the final pellet was obtained and not resuspended. A small amount of each pellet was spotted in 2 spots of a 96-spot MALDI metal plate with the aid of a wooden pick, and 1 μ L of α -cyano-4-hydroxycinnamic acid (HCCA) matrix was pipetted to each spot. After allowing the material in the spots to dry, the plate was scanned and read in a MALDI Biotyper sirius instrument (BD).

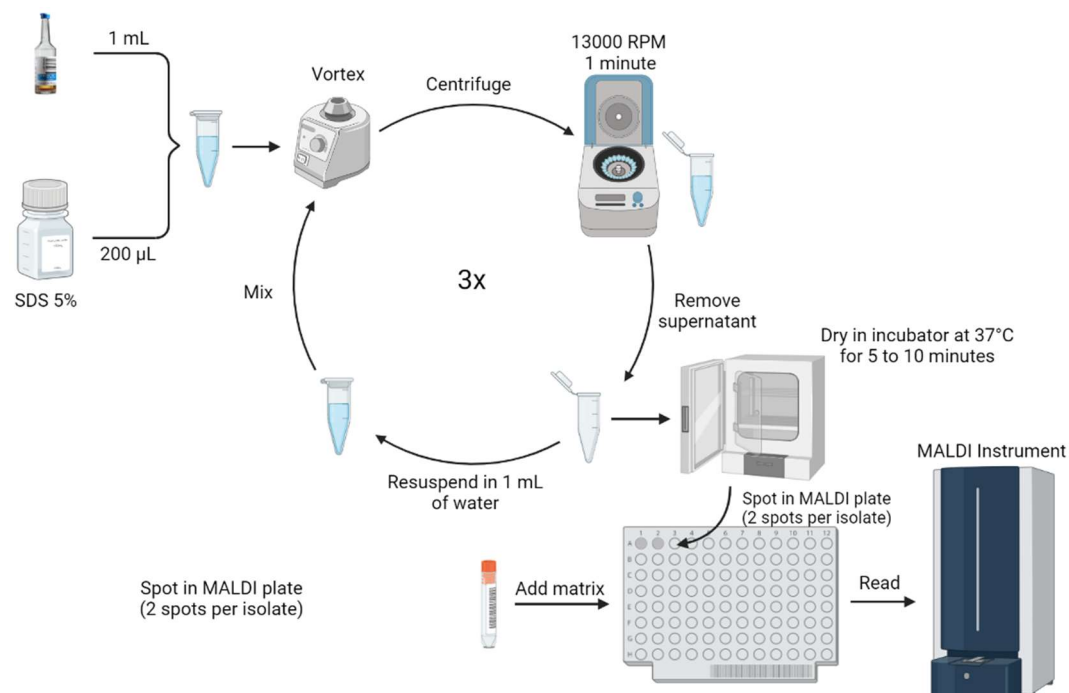


Figure 14: Schematics of the workflow involved in the processing of the sample according to the SDS method for rapid ID by MALDI-TOF MS. Illustration designed on BioRender [52].

FASTinov ID method

The second method was developed by FASTinov, for direct incorporation into the FAST procedure. This method was designed to utilize the same bacteria suspension used in FAST, after being subjected to a cleaning process (Figure 15).

It is performed from the microcentrifuge tube containing the bacterial pellet resuspended in saline solution resultant from the processing of the blood culture for AST.

The tube was centrifuged at 13000 RPM for 1 minute, the supernatant was discarded, and 1 mL of sterile distilled water was added and mixed by vortex. This cycle was repeated one time and the resultant pellet was then dried in an incubator at 37°C, for 5 to 10 minutes. After drying, the pellet was spotted in a MALDI plate, HCCA matrix was added, and the plate was read as described in the SDS method.

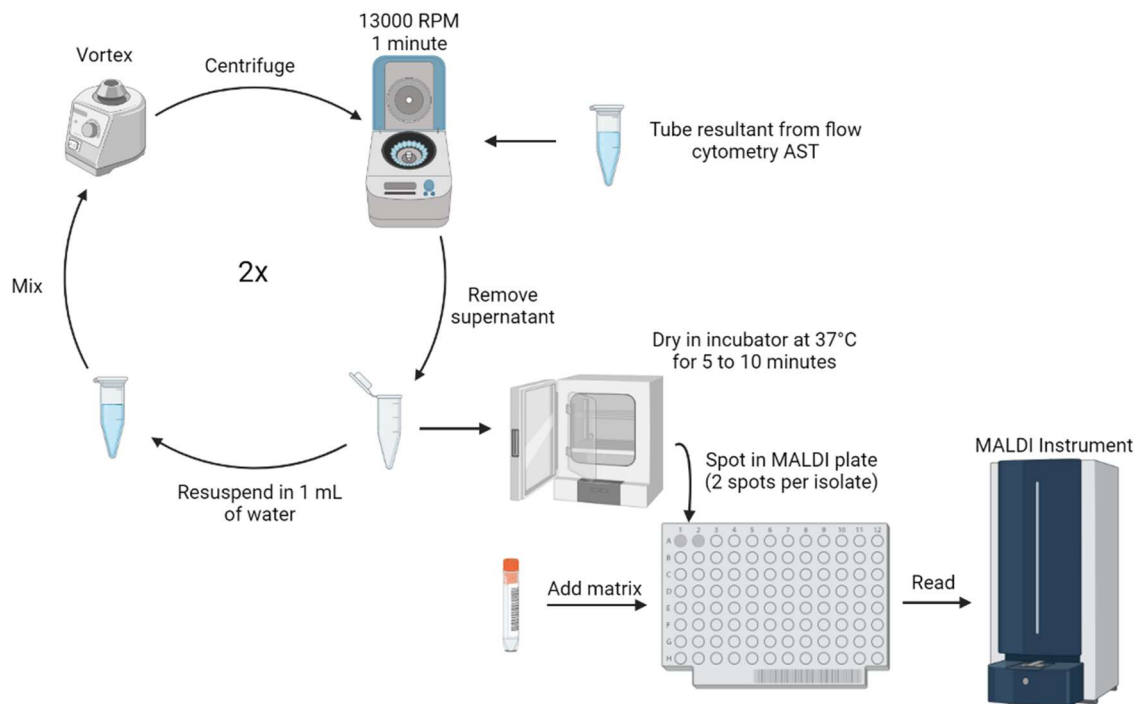


Figure 15: Schematics of the workflow involved in the processing of the sample according to the FASTinov method for rapid ID by MALDI-TOF MS. Illustration designed on BioRender [52].

Conventional ID from bacterial colonies

Conventional ID from bacterial colonies grown overnight was performed as a reference method.

Approximately 1 mL of blood culture was inoculated and streaked in BHI agar medium and incubated at 37°C overnight. One of the resulting colonies was spotted in a MALDI

plate, HCCA matrix was added, and the plate was read as described in the previous methods.

Treatment of data and statistical analyses

Resulting data obtained from FAST and reference AST methods (broth microdilution and disk-diffusion) were organized in Microsoft Excel. Several parameters were calculated to assess the performance of FAST versus reference AST methods: essential agreement (EA – percentage of FAST MIC results within ± 1 serial 1:2 dilution from the reference results, calculated for vancomycin only), categorical agreement (CA – percentage of FAST results that resulted in the same susceptibility category as the reference method), very major errors (VME – percentage of false susceptible results), major errors (ME – percentage of false resistant results), and minor errors (mE – percentage of intermediate results falsely reported as susceptible or resistant, and vice-versa).

Resulting data from MALDI-TOF MS ID (species identification and respective score) were organized in Microsoft Excel. For both direct ID methods, the criteria described in López-Pintor et al. (2019) were used to define a correctly identified isolate: a >1.7 score in at least one of the spots, or a >1.5 score in both spots with the same identified genus and species. The number of correct identifications were used to calculate the percent accuracy (PA) of both direct ID methods in comparison with the colony-based method, which was considered the reference.

Company support

In the scope of this internship, support was provided to the company in several areas.

Scientific and commercial communication – Congress preparation and attendance

Several activities accompanying the launch of the FASTgrampos, FASTgramneg and FASTcolistin kits were performed in order to raise awareness for the company's entry in the AST market. The attendance of scientific/industry congresses was planned and prepared in the scope of this internship. The company's goals with the presence in the congresses were to explain the product's scientific background, to communicate the products' launch to microbiology clinicians and to the industry, to evaluate the sector's interest and to identify potential customers.

Three congresses were identified as relevant for the company in 2022: XI Congresso Nacional de Patologia Clínica (CNPC), from March 17 to 19; the 32nd European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), from May 23 to 26; and 27^o Simpósio de Infecção e Sepsis (SIS), from June 6 to 8.

AST market competitor study

A study of the available AST methods and companies was performed to better educate FASTinov's team on the company's competitors, in preparation for the team members' contact with the industry at ECCMID. Information about reference methods (dilution and diffusion methods), automatic AST methods and rAST methods was compiled using various sources, such as scientific literature, standards documents, and company websites. The more recent novel methods were presented to the team in a meeting before the congress. The resulting information of these efforts was compiled into a small review of rAST tests and included in this report's introduction, in subsection 2.1.4.

Results

Comparison between FAST and reference AST methods

FAST results were presented to include the obtained susceptibility categories, CA (%) and mE, ME and VME, all according to both EUCAST and CLSI standards, for each assessed antimicrobial and for the overall test performance. Results obtained for the FASTgramneg kit were shown for *Enterobacterales* (Table 3), non-fermenters (Table 4) and total gram-negative isolates (Table 5).

Table 3: Results obtained for FASTgramneg kit in comparison with the reference methods, using blood cultures spiked with *Enterobacterales* isolates.

FASTgramneg – <i>Enterobacterales</i> spiked on blood cultures																	
Antimicrobial agent	EUCAST								CLSI								
	RM								RM								
	n	S	I	R	CA (%)	mE	ME	VME	n	S	I	SDD	R	CA (%)	mE	ME	VME
Ampicillin	78	11	-	67	100	-	-	-	78	11	-	-	67	100	-	-	-
Amoxicillin-clavulanic acid	78	16	-	62	100	-	-	-	78	17	5	-	56	96.2	3/78	-	-
Cefotaxime	78	24	-	54	97.4	-	2/24	-	78	24	-	-	54	97.4	-	2/24	-
Ceftazidime	78	23	-	55	97.4	-	1/23	1/55	78	24	1	-	53	98.7	-	-	1/53
Cefepime	78	29	-	49	98.7	-	1/29	-	78	29	-	2	47	98.7	1/78	-	-
Piperacillin-tazobactam	78	31	-	47	96.1	-	-	3/47	78	31	2	-	45	94.9	3/78	-	1/45
Ceftolozane-tazobactam	78	42	-	36	91.0	-	4/42	3/36	78	42	4	-	32	84.6	5/78	3/42	3/32
Ceftazidime-avibactam	78	75	-	3	100	-	-	-	78	75	-	-	3	100	-	-	-
Imipenem	78	74	-	4	100	-	-	-	78	67	7	-	4	100	-	-	-
Meropenem	78	72	-	6	97.4	-	1/72	1/6	78	66	5	-	7	96.2	2/78	1/66	-
Ciprofloxacin	78	36	-	42	98.7	-	1/36	-	78	36	2	-	40	98.7	1/78	-	-
Gentamicin	78	46	-	32	100	-	-	-	78	47	1	-	30	100	-	-	-
Amikacin	78	72	-	6	100	-	-	-	78	74	-	-	4	100	-	-	-
Overall	1014	551	0	463	98.2%	0.0%	1.8%	1.7%	1014	543	27	2	442	97.3%	1.5%	1.1%	1.1%

RM – Reference method

n – number of strains

S – Susceptible; I – Intermediate (or Susceptible for increased exposure, in case of EUCAST); SDD – Susceptible Dose-Dependent; R – Resistant

CA (%) – Percentage of Categorical Agreement

mE – minor Error; ME – Major Error; VME – Very Major Error

Table 4: Results obtained for FASTgramneg kit in comparison with the reference methods, using blood cultures spiked with non-fermenter isolates.

Antimicrobial agent	FASTgramneg – Non-fermenters spiked on blood cultures															
	EUCAST								CLSI							
	RM								RM							
	n	S	I	R	CA (%)	mE	ME	VME	n	S	I	R	CA (%)	mE	ME	VME
Ceftazidime**	72	-	29	43	98.6	-	1/29	-	72	29	2	41	98.6	-	1/29	-
Cefepime**	72	-	39	33	100	-	-	-	72	40	1	31	100	-	-	-
Piperacillin-tazobactam***	72	-	48	24	93.1	-	5/48	-	102	56	4	42	91.2	4/102	5/56	-
Ceftalozane-tazobactam**	72	67	-	5	100	-	-	-	72	67	-	5	100	-	-	-
Ceftazidime-avibactam**	72	67	-	5	100	-	-	-	72	67	-	5	100	-	-	-
Imipenem	102	11	38	53	97.1	-	3/49	-	102	49	2	51	97.1	1/102	2/49	-
Ciprofloxacin	102	-	48	54	100	-	-	-	102	48	1	53	97.1	1/102	2/48	-
Gentamicin*	30	16	-	14	100	-	-	-	102	70	2	30	99.0	1/102	-	-
Amikacin	102	81	-	21	100	-	-	-	102	81	-	21	100	-	-	-
Overall	696	145	134	252	98.7%	0.0%	2.0%	0.0%	798	507	12	279	97.9%	0.8%	1.9%	0.0%

Table legend as per presented in Table 3.

* Not applicable for *Pseudomonas* spp. on EUCAST protocol

** Not applicable for *Acinetobacter* spp. either on EUCAST or CLSI protocols

*** Not applicable for *Acinetobacter* spp. either on EUCAST protocol

Table 5: Total results for gram-negative bacteria obtained for FASTgramneg kit in comparison with the reference methods, using spiked blood cultures.

FASTgramneg – total spiked blood cultures																	
Antimicrobial agent	EUCAST								CLSI								
	RM				CA (%)	mE	ME	VME	RM					CA (%)	mE	ME	VME
	n	S	I	R					n	S	I	SDD	R				
Ampicillin	78	11	-	67	100	-	-	-	78	11	-	-	67	100	-	-	-
Amoxacillin-clavulanic acid	78	16	-	62	100	-	-	-	78	17	5	-	56	96.2	3/78	-	-
Cefotaxime	78	24	-	54	97.4	-	2/24	-	78	24	-	-	54	97.4	-	2/24	-
Ceftazidime	150	23	29	98	98.0	-	2/52	1/98	150	53	3	-	94	98.7	-	1/53	1/94
Cefepime	150	29	39	82	99.3	-	1/68	-	150	69	1	2	78	99.3	1/150	-	-
Piperacillin-tazobactam	150	30	49	71	94.7	-	5/79	3/71	180	87	6	-	87	92.8	7/180	5/87	1/87
Ceftolozane-tazobactam	150	109	-	41	95.3	-	4/109	3/41	150	109	4	-	37	92.7	5/150	3/109	3/37
Ceftazidime-avibactam	150	142	-	8	100	-	-	-	150	142	-	-	8	100	-	-	-
Imipenem	180	85	38	57	98.3	-	3/123	-	180	116	9	-	55	98.3	1/180	2/116	-
Meropenem	78	72	-	6	97.4	-	1/72	1/6	78	66	5	-	7	96.2	2/78	1/66	-
Ciprofloxacin	180	36	48	96	99.4	-	1/84	-	180	84	5	-	93	97.8	2/180	2/84	-
Gentamicin	108	62	-	46	100	-	-	-	180	117	3	-	60	99.4	1/180	-	-
Amikacin	180	153	-	27	100	-	-	-	180	155	-	-	25	100	-	-	-
Overall	1710	792	203	715	98.4%	0.0%	2.5%	1.1%	1812	1050	39	2	721	97.6%	1.2%	1.5%	0.7%

Table legend as per presented in Table 3.

* Not applicable for *Pseudomonas* spp. on EUCAST protocol

** Not applicable for *Acinetobacter* spp. either on EUCAST or CLSI protocols

*** Not applicable for *Acinetobacter* spp. either on EUCAST protocol

Results for the FASTgrampos kit included the same performance parameters, with the addition of EA (%) for vancomycin. These were summarized for *Staphylococcus* spp (Table 6), *Enterococcus* spp. (Table 7), and for total gram-positive isolates (Table 8).

Table 6: Results obtained for FASTgrampos kit in comparison with the reference methods, using blood cultures spiked with *Staphylococcus* spp. isolates.

FASTgrampos – <i>Staphylococcus</i> spp. spiked on blood cultures																		
Antimicrobial agent	EUCAST									CLSI								
	RM				EA (%)	CA (%)	mE	ME	VME	RM				EA (%)	CA (%)	mE	ME	VME
	n	S	I	R						n	S	I	R					
Penicillin*	35	7	-	28	-	94.3	-	2/7	-	66	11	-	55	-	95.5	-	3/11	-
Cefoxitin**	43	26	-	17	-	95.3	-	2/26	-	43	26	-	17	-	95.3	-	2/26	-
Oxacillin***	23	4	-	19	-	91.3	-	2/4	-	23	4	-	19	-	91.3	-	2/4	-
Vancomycin****	66	66	-	-	100	100	-	-	-	66	66	-	-	100	100	-	-	-
Linezolid	66	64	-	2	-	98.5	-	1/64	-	66	64	-	2	-	98.5	-	1/64	-
Gentamicin	66	37	-	29	-	98.5	-	1/37	-	66	51	-	15	-	97.0	1/66	1/51	-
Overall	299	204	-	95	100%	97.3%	0.0%	3.9%	0.0%	330	222	-	108	100%	97.0%	0.3%	4.1%	0.0%

RM – Reference method

n – number of strains

S – Susceptible; I – Intermediate (or Susceptible for increased exposure, in case of EUCAST); SDD – Susceptible Dose-Dependent; R – Resistant

CA (%) – Percentage of Categorical Agreement

EA (%) – Percentage of Essential Agreement

mE – minor Error; ME – Major Error; VME – Very Major Error

* Only for *S. aureus* on EUCAST protocol

** Not applicable for *S. epidermidis*

*** Only for *S. epidermidis*

**** MIC values for *S. aureus*

Table 7: Results obtained for FASTgrampos kit in comparison with the reference methods, using blood cultures spiked with *Enterococcus* spp. isolates.

FASTgrampos – <i>Enterococcus</i> spp. spiked on blood cultures																		
EUCAST										CLSI								
Antimicrobial agent	RM				EA (%)	CA (%)	mE	ME	VME	RM				EA (%)	CA (%)	mE	ME	VME
	n	S	I	R						n	S	I	R					
Penicillin	IE	IE	IE	IE	-	-	-	-	-	65	38	-	27	-	100	-	-	-
Ampicillin	65	42	-	23	-	98.5	1/65	-	-	65	42	-	23	-	100	-	-	-
Imipenem	65	-	39	26	-	100	-	-	-	NA	NA	NA	NA	-	-	-	-	-
Vancomycin*	44	34	-	10	-	95.5	-	2/34	-	44	34	3	7	-	95.5	-	2/34	-
Linezolid	65	64	-	1	-	100	-	-	-	65	64	-	1	-	98.5	1/65	-	-
Gentamicin high level*	47	35	-	12	-	95.5	-	2/35	-	47	35	-	12	-	97.9	-	1/35	-
Overall	286	175	39	72	-	98.2%	0.4%	2.3%	0.0%	286	213	3	70	-	98.6%	0.3%	1.4%	0.0%

RM – Reference method

n – number of strains

S – Susceptible; I – Intermediate (or Susceptible for increased exposure, in case of EUCAST); SDD – Susceptible Dose-Dependent; R – Resistant

CA (%) – Percentage of Categorical Agreement

mE – minor Error; ME – Major Error; VME – Very Major Error

* Only for *E. faecalis*

Table 8: Total results for gram-positive bacteria obtained for FASTgrampos kit in comparison with the reference methods, using spiked blood cultures.

FASTgrampos – total spiked blood cultures																	
Antimicrobial agent	EUCAST									CLSI							
	RM									RM							
	n	S	I	R	EA (%)	CA (%)	mE	ME	VME	n	S	I	R	EA (%)	CA (%)	mE	ME
Penicillin*	35	7	-	28	-	94.3	-	2/7	-	132	49	-	82	-	97.7	-	3/49
Ampicillin	65	42	-	23	-	98.5	1/65	-	-	65	42	-	23	-	100	-	-
Cefoxitin**	43	26	-	17	-	95.3	-	2/26	-	43	26	-	17	-	95.3	-	2/26
Oxacillin***	23	4	-	19	-	91.3	-	2/4	-	23	4	-	19	-	91.3	-	2/4
Imipenem	65	-	39	26	-	100	-	-	-	NA	NA	NA	NA	-	-	-	-
Vancomycin****	110	100	-	10	100	98.2	-	2/100	-	110	100	3	7	100	98.2	-	2/100
Linezolid	131	128	-	3	-	99.2	-	1/128	-	131	128	-	3	-	98.5	1/131	1/128
Gentamicin	66	37	-	29	-	98.5	-	1/37	-	66	51	-	15	-	97.0	1/66	1/51
Gentamicin high level	47	35	-	12	-	95.5	-	2/35	-	47	35	-	12	-	97.9	-	1/35
Overall	585	379	39	167	100%	97.8%	0.1%	3.2%	0.0%	616	435	3	178	100%	97.7%	0.3%	2.8%

RM – Reference method

n – number of strains

S – Susceptible; I – Intermediate (or Susceptible for increased exposure, in case of EUCAST); SDD – Susceptible Dose-Dependent; R – Resistant

CA (%) – Percentage of Categorical Agreement

mE – minor Error; ME – Major Error; VME – Very Major Error

* Only for *S. aureus* on EUCAST protocol

** Not applicable to *S. epidermidis*

*** Only for *S. epidermidis*

**** MIC values for *S. aureus*

MALDI-TOF MS direct ID method comparison

The obtained correct ID numbers were shown for gram-positive and gram-negative pathogens, and for each pathogen genus (Table 9). The PA for each method was determined in comparison with the reference method as a performance indicator. Results were presented for gram-positive, gram-negative, and overall results. The obtained overall PA (%) was 83.13% for the FASTinov method, and 67.47% for the SDS method.

Table 9: Number of correct IDs obtained for each method as well as the determined PA (%), for each genus of gram-positive and gram-negative pathogens, and overall identifications.

Gram-positive	Total number of IDs (reference)	FASTinov method		SDS method	
		Correct ID	PA (%)	Correct ID	PA (%)
<i>Staphylococcus spp.</i>	39	34	87.18	25	64.10
<i>Enterococcus spp.</i>	3	1	33.33	2	66.67
Total gram-positive	42	35	83.33	27	64.29

Gram-negative	Total number of IDs (reference)	FASTinov method		SDS method	
		Correct ID	PA (%)	Correct ID	PA (%)
<i>Escherichia spp.</i>	22	21	95.45	19	86.36
<i>Klebsiella spp.</i>	14	9	64.29	8	57.14
<i>Pseudomonas spp.</i>	3	2	66.67	1	33.33
<i>Morganella morganii</i>	1	1	100.00	0	0.00
<i>Proteus mirabilis</i>	1	1	100.00	1	100.00
Total gram-negative	41	34	82.93	29	70.73

Overall	83	69	83.13	56	67.47
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In order to better understand the impact of incorrect identifications, additional parameters were calculated: percentage of nonidentifications (number of samples for which no identification was able to be provided by the MALDI-TOF MS system), misidentifications (number of samples for which an identification was provided that did not agree with the reference method), and no-score identifications (number of samples for which at least one of the spots was able to be identified, but was regarded as incorrect due to not upholding the score value standards established in the criteria) (Tables 10 and 11). Note the very low number of cases of misidentification in both methods.

Table 10: Overall number and percent values of nonidentifications, misidentifications, and no-score identifications, as well as the total number of wrong identifications, for each assessed method.

Overall		FASTinov	SDS
Nonidentification	Number	14	20
	Percent (%)	16.87	24.10
Misidentification	Number	0	1
	Percent (%)	0.00	1.20
No-score	Number	0	6
	Percent (%)	0.00	7.23
TOTAL	Number	14	27
	Percent (%)	16.87	32.53

Table 11: Number and percent values of nonidentifications, misidentifications, and no-score identifications, as well as the total number of wrong identifications, for gram-positive and gram-negative samples, for each assessed method.

Gram-positive		FASTinov	SDS	Gram-negative		FASTinov	SDS
Nonidentification	Number	7	11	Nonidentification	Number	7	9
	Percent (%)	16.67	26.19		Percent (%)	17.07	21.95
Misidentification	Number	0	1	Misidentification	Number	0	0
	Percent (%)	0.00	2.38		Percent (%)	0.00	0.00
No-score	Number	0	3	No-score	Number	0	3
	Percent (%)	0.00	7.14		Percent (%)	0.00	7.32
TOTAL	Number	7	15	TOTAL	Number	7	12
	Percent (%)	16.67	35.71		Percent (%)	17.07	29.27

Preparation of promotional material and scientific congresses

CNPC is a yearly Portuguese congress organized by the Portuguese Society for Clinical Pathology. The XI edition, which FASTinov attended, occurred from March 17 to 19 at Culturgest Lisboa. The company had a small publicity space, which allowed for a table and two chairs (Figure 16). Two 80 x 200 cm rollups with information about the product and its workflow were designed and printed to serve as informative pieces of decoration. Two divulgation posters were also prepared (one referring to a previous external validation study performed in Hospital Ramón y Cajal in Madrid and the other referring to an internal validation of the use of the FASTcolistin MIC kit for urines, blood cultures and colonies) and were printed in A4 format and delivered to congress attendees. I participated in the rollup design and print order, as well as in the poster design.



Figure 16: Photo of FASTinov's publicity space at CNPC, in Culturgest Lisboa, Lisbon, Portugal.

At ECCMID, the company was provided with a 4 x 3 m enclosed publicity booth (Figure 17). The front of the space had a table with a TV screen looping a PowerPoint presentation of the company and its products. The previously prepared posters were also printed in 100 x 150 cm format as PVC banners to present scientific data related to the product and to decorate the booth's side walls. The two rollups were also used as decoration and as a divider of the front and rear part of the booth, in order to hold meetings with potential customers at the rear, in the second table. In order to facilitate conversations with potential customers, printouts were designed with relevant information such as the antibiotic panel of each kit, the procedure's workflow and clinical timeline. I participated in the overall booth design, the preparation of the PowerPoint presentation loop, the poster design and print order, the printout design, and the ordering of additional furniture at the ECCMID shop. I also had the opportunity to personally attend the congress in its third day (March 25).



Figure 17: Photo of FASTinov's publicity booth during ECCMID, at Altice Arena, in Lisbon, Portugal.

Discussion

Although a reduction in antimicrobial consumption can appear to be the obvious solution to the problem, it should not be the only one, as its efficacy might vary from region to region. For example, limiting access to antimicrobials in South Asia might be helpful as the main drivers of AMR in the region seem to be overuse and misuse; however, one might argue that an increase in ease of access to antimicrobials would alleviate the AMR burden in regions with poor access to second-line antibiotics, such as western sub-Saharan Africa [19]. AMR is a complex issue and, as such, must be tackled through several appropriate strategies.

To correctly implement antimicrobial stewardship programs through the de-escalation of antimicrobial use, it is essential to provide an infrastructure that allows the accurate and timely diagnosis of infections, for which rapid ID and rAST have an immense potential. The increase of interest in the implementation of novel technologies in the diagnostic procedure can explain the vast expansion of the rAST market, which currently encompasses many emerging companies, with some of them already available on the market. There is immense variability among the rAST market pertaining to the technology used, the sample preparation method, the time-to-results, the validated strain-drug combinations, the number of samples for each run, and the capability of performing ID. This variability confers different advantages and limitations to each test, which each clinician can analyse to choose the alternative that better suits the logistics and needs of their respective laboratory or health centre. For this reason, it's hard to admit that a single test is better or worse than the others, as each alternative will most likely find a place in the market.

Despite the differences, there is one parameter which seems to be admitted as a core importance by most, if not all companies involved in the market: speed. The reduction in time-to-results is a goal that is shared by every company due to its importance in improving clinical outcomes, and as such is a major advertisement point. The improvements in time-to-results made possible by FAST technology remain unprecedented when compared to competitors which are already present in the market, as no other established technology can reach a time-to-results of under 2 hours.

Besides the advantage in reduction of time-to-results, FAST's performance indicators are according to standards, as observed in this work. In the case of gram-negative bacteria, the obtained CA (%) was $\geq 98.4\%$ considering EUCAST breakpoints and $\geq$

97.3% considering CLSI breakpoints. Both values met the standard minimum for this parameter ($\geq 90\%$). All the obtained error values did not exceed the usual established limits for performance (ME and $VME \leq 3.0\%$).

In the case of gram-positive bacteria, all the obtained CA (%) values were also according to standards. The obtained EA (%) for vancomycin was 100% considering both EUCAST and CLSI protocols, well above the standard minimum value for this parameter ($\geq 90\%$). The obtained VME (%) value considering both genera and total gram-positive bacteria was 0.0% according to both breakpoint protocols. ME (%) values exceeded the established limit for *Staphylococcus* isolates, which consequently resulted in a slightly exceeded value of 3.2% in the case of EUCAST breakpoints for total gram-positive isolates. This might have been due to small sample size, which can consequently result in overestimated error percentages. Overall, general performance indicators of FASTgrampos and FASTgramneg in this study were according to standards, as was described in previous internal and external validations of the products.

FASTinov's commitment to speed is not limited to the reduction in AST time-to-results, however. As previously stated, all procedures involved in the workflow of BSI/sepsis diagnosis are plagued by slow turnaround times, and pathogen ID is no exception. Short subculturing is a viable alternative to eliminate overnight incubation but is still not the ideal option. The development of a method that allows for the performance of MALDI-TOF MS ID directly from positive blood cultures would further decrease the time until an identification is obtained, which is essential when the application of a very fast AST method such as FAST is introduced into the workflow. For this reason, the performance of two different direct ID methods, one of which developed by FASTinov and designed to be incorporated with FAST itself, were compared to the reference standard for MALDI-TOF MS ID.

In this work, the FASTinov method generally performed better than the SDS method, as observed by its higher total PA (83.13% versus 67.47%, respectively). The FASTinov method performed better especially in gram-positive isolates, as the difference in PA was higher in this case than in the case of gram-negative isolates. The obtained PA values for both of the assessed methods were considered to be somewhat suboptimal, as failure to provide an accurate ID is not uncommon when compared to the reference method. In spite of this, the analysis of the cases for which the methods did not provide a correct ID has shown that the observed total percentage of misidentifications is very low (1.20%), with only one observed case of misidentification detected in the SDS method. This indicates that it is very unlikely that a failed ID will produce a misidentified species result,

as the most common case of failure results in no ID being provided whatsoever. From this perspective, the usefulness of these methods increases despite their relevant percentage of failed identifications, because if a species-level ID is produced, there is a low chance of it being incorrect, and thus a low probability of inducing the clinician in error. Additionally, in the case of a specific gram-positive isolate, the FASTinov method was able to discern two different species in the two inoculated spots, one of them being in agreement with the reference method and the other one being identified as a different bacillus. Although this could be a case of misidentification, it could also be a successful identification of a mixed culture that was overlooked in the reference method, however, this was not confirmed by culturing the isolate.

As this was a preliminary study, additional assessments of both ID methods should be performed in the future to better appraise their performance and usefulness in a clinical setting. However, the results obtained in this work are enough to justify the immense potential in MALDI-TOF MS pathogen ID directly from bacterial pellets obtained from blood cultures, and its incorporation into the workflow of FAST. The implementation of both direct ID and FAST can substantially decrease the time until a susceptibility report is generated for cases of BSI and sepsis (Figure 18).

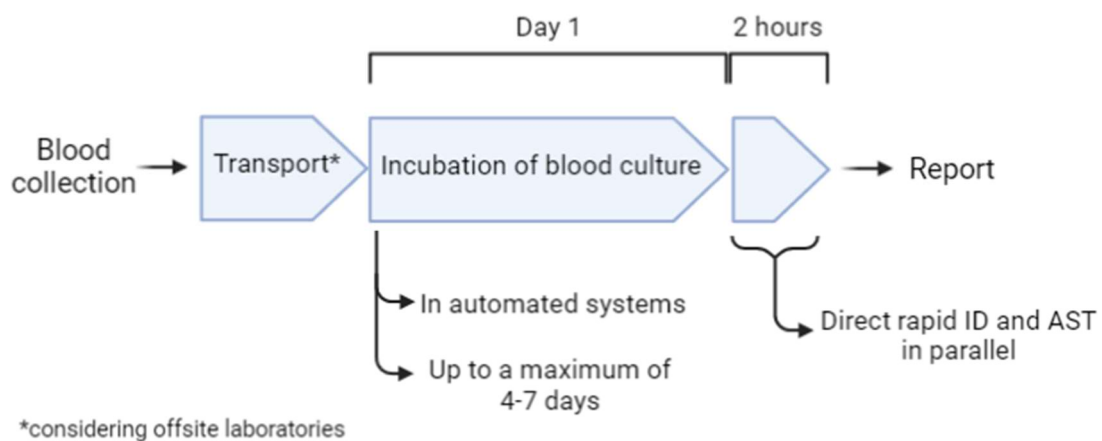


Figure 18: Schematic representation of the proposed approach of implementation of direct rapid ID by MALDI-TOF MS and FAST on BSI/sepsis diagnosis, including timeframes of the performed procedures. Timeframe not to scale for illustration purposes. Illustration designed on BioRender [52], and adapted from (Lamy et al., 2020)[53].

On a personal level, the internship at FASTinov has provided a very different experience than the standard master's degree dissertation. This internship allowed me to experience work in an ISO 9001 certified laboratory and participate in the procedures for the compliance with the associated regulation, such as temperature and humidity control,

periodic cleaning, and equipment maintenance. In addition to laboratory work, I was incentivized to develop other skillsets that would normally not be acquired in an academic context through the participation in some commercial aspects of the company. The possibility of working with collaborators with different backgrounds such as business administration, and the participation in the preparation and attendance of scientific and commercial congresses have provided me with a better understanding of the industrial aspect of biomedical science, as well as the intricacies of the regulations associated with the launch of an *in vitro* diagnostic product, which I considered invaluable information.

Conclusion

In conclusion, the work performed as a result of this internship provided an additional assessment of the performance of two of FASTinov's AST kits, which produced overall results in agreement with the required standards for novel AST methods: CA (%) and EA (%) $\geq 90\%$, VME and ME $\leq 3.0\%$, with the exception of ME, which slightly exceeded the limit (3.2%) in the case of gram-positive bacteria considering the EUCAST protocol, which can be due to the small sample size of the study. This re-emphasized the usefulness of FAST as a viable alternative to reference AST methods, not only due to its unprecedented time-to-results of only 2 hours, but also due to its accuracy and precision.

In addition, the performance of two pellet-based methods of performance of MALDI-TOF MS ID directly from blood cultures was assessed in comparison to samples obtained from bacterial colonies grown overnight and, although the obtained overall PA values were not ideal, the observed very low percentage of misidentifications confers confidence to the ID results obtained from both methods. Although this was a preliminary study, the obtained results were promising and additional assessments of performance will be likely performed in the future due to their potential usefulness in further reducing the time-to-results of BSI/sepsis diagnosis, and their incorporation as rapid ID methods complementary to FAST or other rAST methods.

My time at FASTinov has allowed me to gain experience in areas I would generally not be able to contact in an academic research context, for which I considered this internship very rewarding on a personal and academic level.

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Appendix

Supplementary Table 1: Utilized reference strains obtained from bacteria strain collections, with their respective species and designation.

Gram-negative		Gram-positive	
Species	Designation	Species	Designation
<i>Escherichia coli</i>	ATCC 25922	<i>Staphylococcus aureus</i>	ATCC 29213
<i>Escherichia coli</i>	ATCC 8739	<i>Staphylococcus aureus</i>	ATCC 43300
<i>Escherichia coli</i>	ATCC 35218	<i>Staphylococcus aureus</i>	ATCC 700698
<i>Escherichia coli</i>	BAA-2425	<i>Staphylococcus epidermidis</i>	ATCC 35984
<i>Klebsiella pneumoniae</i>	ATCC 13443	<i>Enterococcus faecalis</i>	ATCC 29212
<i>Klebsiella pneumoniae</i>	BAA-1705	<i>Enterococcus faecalis</i>	ATCC 51299
<i>Klebsiella pneumoniae</i>	ATCC 700603	<i>Enterococcus faecium</i>	ATCC 700221
<i>Klebsiella pneumoniae</i>	BAA-1706	<i>Enterococcus casseliflavus</i>	ATCC 700668
<i>Enterobacter cloacae</i>	CCUG 59627	<i>Enterococcus gallinarum</i>	ATCC 49608
<i>Enterobacter aerogenes</i>	ATCC 13048		
<i>Serratia marcescens</i>	ATCC 14756		
<i>Providencia rettgeri</i>	BAA-2525		
<i>Pseudomonas aeruginosa</i>	ATCC 27853		
<i>Pseudomonas aeruginosa</i>	BAA-2108		
<i>Acinetobacter baumannii</i>	BAA-1709		