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Evaluation of new strategies to improve bloodstream infections diagnostic workflow and Antimicrobial Stewardship

Tutor:

Prof. Rossana Cavallo

Candidate:

Dr. Antonio Curtoni

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Index

Abstract

Improving blood culture (BC) turn-around time (TAT) is pivotal in the management of septic patients. Faster reporting of identification (ID) and antimicrobial susceptibility testing (AST) results can significantly impact on patients outcomes and costs associated with inappropriate antimicrobial therapy. Unfortunately, BC TAT of conventional phenotypic conventional methods is far from optimal patients management since 24-72 hours are needed to reach a complete microbiological report with ID and AST. At the Teaching Hospital *Molinette* of the *A.O.U. Città della Salute e della Scienza di Torino*, a preliminary retrospective epidemiological evaluation was assessed to set-up a rapid protocol (RP) for bloodstream infections (BSI) diagnosis and an observational study was conducted to assess its potential clinical impact. The RP was characterized by the presence of 2 combined technologies, Light Scatter Technology (LST) and MALDI-TOF MS for direct AST and ID of the aetiologic agent of BSI from positive bacterial monomicrobial BC. The positive BC have been prospectively screened, and 102 septic episodes considered. Each sample was processed according to the standard protocol (SP) performed with conventional reference methods and the RP. RP accuracy and TAT were evaluated and compared to SP. To assess the potential impact of the RP results on the antimicrobial therapy management, clinicians were presented with the RP and SP results and interviewed on therapeutic decisions. Overall AST concordance between RP and SP was 94.8% (Cohen K 0.85: almost perfect agreement). The RP technical TAT was lower than SP (6.4h *vs.* 18.4h), and even more so was the real-life TAT (average advantage 21.1h 90% CI 20.5-21.8). The concordance between RP-based and SP-based antimicrobial therapy decisions was 90.9% (90% CI 84.7-96.2). According to RP results there would have been 24.2% correct antibiotic therapy changes one working day earlier with 17.2% of possible de-escalation in antimicrobial therapy regimens. Moreover, in 24.2% of the cases, an early infection control policies application could have been possible. The data suggest that rapid reporting of ID and AST results could benefit more than 1 out of 5 patients thanks to changes of the empirical antibiotic therapy to a targeted one with one working day in advance.

Abbreviations

AS: Antimicrobial Stewardship

AST: Antimicrobial Susceptibility Testing

BC: Blood cultures

BSI: Bloodstream Infection

CI: Confidence Interval

CML: Clinical Microbiology Laboratory

CoNS: Coagulase-Negative Staphylococci

CPE: Carbapenemase-Producing Enterobacteriaceae

DSP: Definitive Standard Protocol

ESBL: Extended-Spectrum Beta-Lactamases-producers

ID: Identification

LST: Light Scattering Technology

MALDI-TOF: Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry

MDRO: Multidrug-Resistant Organisms

ME: Major error

MIC: Minimum Inhibitory Concentration

miE: Minor Error

MR: methicillin-resistant

p: percentile

POCT: Point-Of-Care Test

PSP: Preliminary Standard Protocol

RP: Rapid Protocol

SE: Sensitivity

SP: Specificity

St.dev.: standard deviation

TAT: Tun-Around Time

VME: Very Major Error

VRE: Vancomycin-Resistant Enterococcus

Introduction

Bloodstream infections and sepsis

Bloodstream infections (BSI) are associated with high morbidity and mortality rates and constitute a serious growing Public Health issue worldwide[1–4]. Affecting approximately 1.2 million people in Europe each year, BSI are a major cause of sepsis, a frequent life-threatening syndrome with an in-hospital mortality rate over 10%[5,6]. Even if the incidence in the developed world of BSI and sepsis is underestimated, it is still growing due to the increasing trend of several risk factors such as population ageing, comorbidities, high-risk surgery in elderly age groups, and selection of multidrug-resistant microorganisms (MDRO) with multiple virulence factors[2,7,8]. Moreover, BSI and sepsis represent an important diagnostic challenge for both physicians and clinical microbiologists[9]. In 2017, sepsis was recognized as a global health priority by the World Health Assembly that developed a resolution to reduce the burden of sepsis by improving its prevention, diagnosis, and management[10]. Nevertheless, the real burden of sepsis, as well as its true incidence, are currently underestimated. A systematic review published in 2016 inferred the global burden from national and local population data, estimating 30 million episodes and 6 million deaths per year[8]. The main reasons underlying these uncertain data are the lack of a gold standard diagnostic test, the evolution of different definitions and diagnostic criteria over time, the reporting mechanism and healthcare workers skills to correctly recognize sepsis. In 2016, the definition of sepsis was revised and updated to its 3rd version (Sepsis-3) by an International Committee convened by the Society of Critical Care Medicine and the European Society of Intensive Care Medicine, The Sepsis Definition Task Force[11]. Sepsis-3 was defined as a "*life-threatening organ dysfunction caused by a dysregulated host response to infection. For clinical operationalization, organ dysfunction can be represented by an increase in the Sequential [Sepsis-related] Organ Failure Assessment (SOFA) score of 2 points or more, which is associated with an in-hospital mortality greater than 10%.*"[11].

Table 1 - Sepsis-2 and Sepsis-3 (qSOFA) definition criteria[5,12,13]

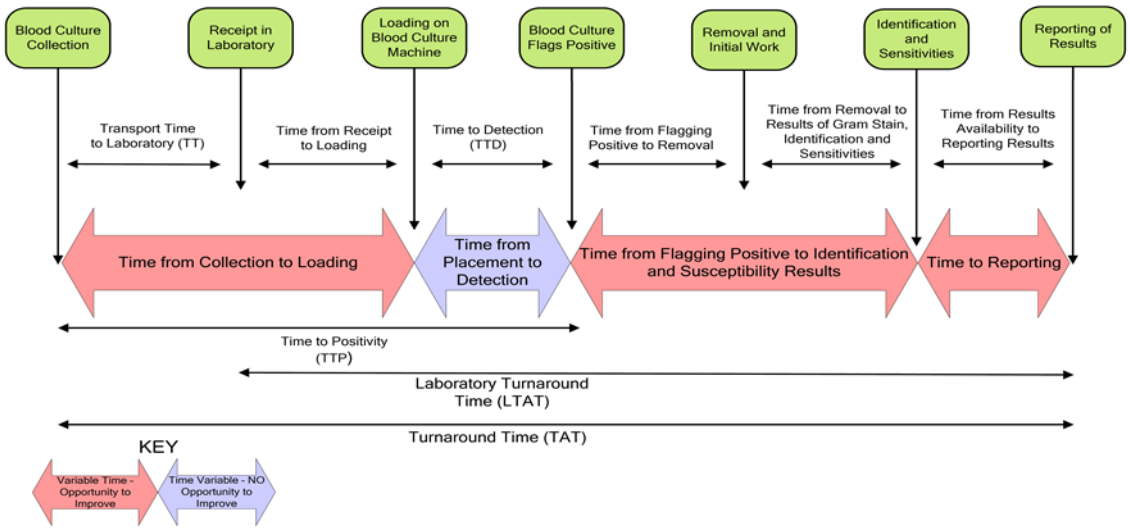
Sepsis-2 **SIRS criteria (≥2)**	**Sepsis-3** **qSOFA criteria (≥2)**
Body temperature >38°C or <36°C	Systolic blood pressure ≤100 mmHg
Respiratory rate > 20 breaths/min or $PaCO_2$ <32 mmHg	Respiratory rate ≥ 22 breaths/min
Heart rate > 90 bpm	Glasgow Coma Scale ≤14
White blood cell count >12000/mm^3 or <4000/mm^3 or >10% immature bands	

The use of the previous definition (Sepsis-2) was considered nonspecific because it is not necessarily associated with a dysregulated life-treating response, while at the same time including many critical patients even in absence of infection[9,11]. The sepsis guidelines identify early and appropriate antimicrobial therapy, as well as early recognition of the syndrome, adequate haemodynamic support and adequate infection source control, among the pillars of effective therapy[11,14].

Bloodstream infections microbiological diagnosis

Blood cultures (BC), though being introduced in the early 20th century, still represent the microbiological gold standard diagnostic test for bloodstream infections (BSI) detection: they allow to confirm the infectious aetiology, isolate and identify the pathogen and guide the antimicrobial therapy, the most important therapeutic measure for patients outcome[15–18]. A timely microbiological report of the aetiological agent of BSI significantly impacts on patients morbidity and mortality rates, moreover, due to the remarked effect on antimicrobials administration and, consequently, on antimicrobial resistance selection and spread, BC are considered a milestone in all the Antimicrobial Stewardship (AS) programmes. Unfortunately, the conventional diagnostic process based on blood cultures, still requires at least 1 to 3 days to achieve microorganism identification (ID) and antimicrobial susceptibility testing (AST) results[18–22].

Figure 1 - Timeline of BSI diagnostic workflow (from UK Standards for Microbiology Investigations: Investigation of blood cultures, 2019)[18]



Conventional reference methods, indeed, even if supported by automated and refined technological platforms that have reduced labour and incubation times, are based on phenotypical tests. By this way, both microorganisms ID and AST must start from isolated colonies obtained from overnight incubation of sample seeded agar plates and require another 18-20 hours to reach final results.

From a national survey on BC turn-around time (TAT) performed in 2015, the median time for microorganism ID by phenotypic methods was of 23.5 hours, while for phenotypic AST results a median of 41.2 hours was required[23].

Table 2– Blood cultures turn-around time analysis (data expressed in hours from the first Italian survey, Arena et al. 2015)[23]

	Check-Pos	Pos-Gram	Pos-ID	Pos/IDM	Pos-ATBM	Pos-ATB
Number of values	302	302	222	80	80	277
Range	1.7-109.4	0.2-86.4	1-132	1-38.4	1-41.5	9.6-138.4
Median	15.1	7.6	23.5	7.9	8.6	41.2
Mean	20.4	10.3	28.7	12	13.2	47.6

Check-Pos, Check-in to positivity; Pos-Gram, positivity to Gram-stain; Pos-IDM, positivity to molecular pathogen identification; Pos-ID, positivity to pathogen identification; Pos-ASTM, positivity to molecular detection of resistance markers; Pos-AST, positivity to phenotypic antimicrobial susceptibility testing.

The TAT performance of microbiological conventional tests unfortunately produces a delay of 18-48 hours for the beginning of a targeted antimicrobial therapy with a significant impact on morbidity and mortality rates, episodes of nosocomial infections caused by MDRO and in antimicrobials related cost[24–27].

Genetic target-based methods have been used to reduce the time to response as regards for both pathogens ID and AST at two different stages in the BSI diagnostic process (Figure 2)[28,29]. The first starting point is directly from the collected patients whole blood: in this case the TAT has the best time gain because the test can be performed directly from blood without waiting the 24-72 hours in which >95% of BC often became positive for clinically significant isolates[30]. The final TAT of these tests is, in most cases, <8 hours, formally configuring them as rapid methods[31,32]. Some of the most widespread commercially available kits and platforms with related sensitivity (SE), specificity (SP) and TAT are reported in Table 3[29].

Table 3 - Commercially available molecular methods for microorganisms ID and AST directly from whole blood (from Peker et al. 2018)[29]

Assay	Pathogens covered	Resistance markers	Sensitivity/ specificity	Complexity -Personnel experience level -Equipment requirement	Hands-on time	Times to result
Nucleic-acid amplification-based methods						
The LightCycler® SeptiFast (Roche Molecular System, Switzerland)	19 Bacteria 5 Candida spp. and *Aspergillus fumigatus*	*mec*A	83–90%/73%	Automated -Trained personnel -Proprietary equipment	3 hours	6 hours
Magicplex™ Sepsis Real-time Test (SeeGene, Korea)[a]	>90 pathogens 27 pathogens at species level	*mec*A, *van*A, *van*B	37–65/66–92%	Multi-step automated -Trained personnel -Proprietary equipment	Not provided	3–6 hours
SepsiTest™ (Molzym, Germany)[a]	345 bacteria, *Candida*, *Cryptococcus* and *Aspergillus* species	None	87%/86%	Partially automated -Trained personnel -Generic equipment	75 min (PCR) 70 min (sequence analysis)	8–12 hours
T2 Magnetic resonance-based methods						
T2Candida Panel (T2Biosystems, USA)	5 *Candida* species	None	99%/91%	Fully automated -No trained personnel -Proprietary equipment	5 min	3–5 hours
Metagenomics						
iDTECT™ Dx Blood (PathoQuest SAS, France)[a]	>1200 bacteria and viruses	None	Not provided	Not provided -Trained personnel -Generic equipment but proprietary software	Not provided	Not provided

Specifications of the assays were collected from the literature and manufacturer's information.
[a] Not available in the USA.

The SE of these approaches is not invalidated by the number of collected BC sets or by insufficient blood volume withdrawal, making them more suitable for paediatric patients application and in the case of previously administrated antimicrobial therapies cause the targets are not viable microbial cells but nucleic acids[27,29,33]. Moreover, they can also be used in polymicrobial blood cultures for detecting more targets simultaneously.[27,29] The second starting point for molecular methods, in the BSI diagnostic process, begins from positive BC bottles. Some of the most frequently used commercially available tests are reported in Table 4 with related SE, SP and TAT. The TAT of these tests is usually <4 hours configuring them as ultra-rapid tests[32]. The single-sample cartridge test design, with few and simple manual steps, makes some of them suitable for point-of-care tests (POCT) especially in hub and spoke hospital logistic organizations.

Figure 2 - Methods to identify microorganisms from blood or positive blood cultures (from Peker et al. 2018)[29]

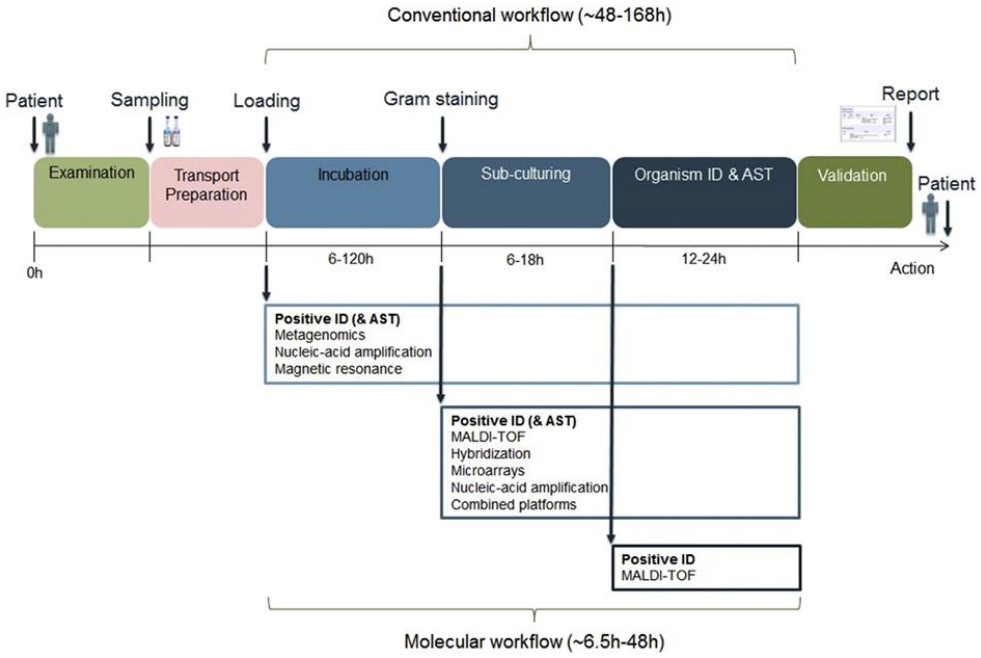


Table 4 - Commercially available molecular methods for microorganisms ID and AST directly from positive BC (from Peker et al. 2018)[29]

Assay	Gram staining need	Pathogens covered (Panel/kit)	Resistance markers	Sensitivity/specificity	Complexity -Personnel experience level -Equipment requirement	Hands-on time	Times to result
***In situ* hybridization-based methods**							
PNA-FISH® (OpGen®, USA)	Yes	4 Gram-positive bacteria (*S. aureus*/CNS PNA-FISH® and *E. faecalis*/OE PNA-FISH®) 3 Gram-negative bacteria (Gram-Negative PNA-FISH®) 5 *Candida* spp. (*Candida* PNA-FISH®, Yeast Traffic Light® PNA-FISH)	None	97-100%/90-100%	Not automated -Trained personnel -Proprietary equipment	10 min	2.5 hours
*Quick*FISH® (OpGen®, USA)	Yes	4 Gram-positive bacteria (*Staphylococcus Quick*FISH®, *Enterococcus Quick*FISH®) 3 Gram-negative bacteria (Gram-Negative *Quick*FISH®) 3 *Candida* spp.[a] (*Candida Quick*FISH®)	None	97–100%/90–100%	Not automated -Trained personnel -Generic equipment	5 min	30 min
AccuProbe® (Hologic®, USA)	Yes	*S. pneumoniae* (*S. pneumoniae* culture identification test) *S. aureus* (*S. aureus* culture identification test)	None	>97%/81–100%	Not automated -Trained personnel -Generic equipment	5 min	1 hour
Accelerate PhenoTest™ BC (Accelerate Diagnostics™, USA)	No	15 Gram-positive bacteria 11 Gram-negative bacteria 2 *Candida* spp. (Accelerate PhenoTest™ BC Kit)	20 antimicrobial MICs determined	96%/99%	Fully automated -No trained personnel -Proprietary equipment	2 min	1 hour ID 6 hours AST
DNA-Microarray based methods							
Verigene® (Luminex® Corporation, USA)	Yes	12 Gram-positive bacteria (VERIGENE® Gram-Positive Blood Culture Test) 9 Gram-negative bacteria (VERIGENE® Gram-Negative Blood Culture Test)	*mec*A, *van*A, *van*B, bla_{NDM}, bla_{VIM}, bla_{KPC}, bla_{OXA}, bla_{CTXM}	81–100%/> 98%	Fully automated -No trained personnel -Proprietary equipment	5 min	2.5 hours
Nucleic-acid amplification-based methods							
FilmArray® (bioMérieux, France)	No	8 Gram-positive bacteria 11 Gram-negative bacteria 5 *Candida* spp. (FILMARRAY® BCID Panel)	*mec*A, *van*A, *van*B, bla_{KPC}	>96%/98–100%	Fully automated -No trained personnel -Proprietary equipment	2 min	1 hour
Xpert® MRSA/SA BC (Cepheid®, USA)	Yes	*S. aureus*, MRSA	*mec*A	98–100%/99–100%	Fully automated -No trained personnel -Proprietary equipment	1 min	1 hour
BD Max™ StaphSR Assay (BD Diagnostics, Canada)	Yes	*S. aureus*, MRSA, CoNS	*mec*A, and *mec*C	98–100%/98–100%	Fully automated -Trained personnel -Proprietary equipment	1 min/specimen	2.5 hours
Eazyplex® MRSA (Amplex Diagnostics GmbH, Germany)[b]	Yes	*S. aureus*, *S. epidermidis* (eazyplex ® MRSA)	*mec*A, and *mec*C	100%/98%	Fully automated -Trained personnel -Proprietary equipment	2 min sample preparation	30 min

Combined methods (Multiplex PCR and hybridization based methods)							
Sepsis Flow Chip (Master Diagnóstica, Spain)[a]	No	>36 bacteria *Candida* spp (non-albicans). *Candida albicans* (Sepsis Flow Chip Kit)	20 antimicrobial resistance markers	93–94 %/100%	Fully automated -Trained personnel -Proprietary equipment	Not provided	3 hours
ePlex® BCID (GenMarkDx®, USA)[a]	Yes	20 Gram-positive bacteria (BCID-GP Panel) 21 Gram-negative bacteria (BCID-GN) 16 yeasts (including 10 *Candida* spp.) (BCID-FP Panel)	BCID-GP: *mec*A, *mec*C, *van*A, *van*B BCID-GN: bla_{CTXM}, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, bla_{OXA}	Not provided	Fully automated -No trained personnel -Proprietary equipment	2 min	1.5 hours

Specifications of the assays were collected from the literature and manufacturer's information.
[a] Not available in the USA.

In spite of TAT performance, molecular methods have several drawbacks in comparison to phenotypic tests. Firstly, the SE, though high, is not comparable with conventional BC methods: molecular targets are limited, and false negative results occur when the aetiological agent of BSI is not covered by the kit's panel. On the other hand, false positive results, caused by the high likelihood of DNA-contamination during blood sampling, are frequent and difficult to correctly recognize due to the lack of other important information such as the number of BC sets or the time to positivity available with conventional reference methods[33,34]. Moreover, the provided information on the antimicrobial pattern of resistance of the detected microorganisms are limited in comparison to phenotypic tests. These tests provide data only on the presence or absence of a few number of resistance genes from which the antimicrobial resistance to selected categories of drugs may be inferred (*e.g.*, bla_{KPC} for carbapenems). In case of positivity for the target gene, the likelihood that the antimicrobial category is resistant is high, even if not certain (the gene could be unexpressed or non-functional), but, of note, in case of negativity there is no assurance of susceptibility of the selected molecules due to the possible co-occurrence of other resistance mechanisms[35,36]. At last, molecular methods heavily impact on CML personnel workload, on organization and budget: usually, the staff involved in test execution should be well trained in Molecular Biology and the CML should be equipped with dedicated expensive and/or bulky instruments[33,34]. A summary of the pros and cons of these methods is reported in Table 5 and Table 6.

Table 5 - Methods applied directly to whole blood: advantages and limits (from Peker et al. 2018[29])

	Blood culture	Nucleic-acid amplification based methods	T2 Magnetic resonance based methods	Metagenomics
Duration[a]	Very slow	Fast or very fast	Slow	Very slow
Complexity	Laborious	Simple	Simple	Laborious
Role	Not applicable	On-top assay	On-top assay	On-top assay
Limits of detection	1–10 CFU/mL	3–30 CFU/mL	1 CFU/mL	Possibly similar to nucleic acid amplification-based methods, depending on the depth of sequencing
Quantification possibilities	Yes (conditionally: time to positivity)	Possible	Not possible	Semi-quantitative
Suitability for therapy monitoring	Yes (bacteraemia)	Potentially (conditionally; indirectly via quantification)	No	Potentially (conditionally; indirectly via quantification)
Costs[b]	<10€ (if negative), 10–50€ (if positive)	50–250€	150–200€	150–300€
Laboratory type (setting)	Satellite	Standard	Centralized or academic	Centralized or academic
Limitations	Very specialized technicians	False-positive results from artefacts	Does not provide information on antifungal resistance	Very specialized technicians

[a] Four categories were considered: Very slow (6–48h); Slow (3–6h); Fast (1–3h); Very fast (<1h).

Table 6 - Methods applied to positive BC: advantages and limits (from Peker et al. 2018[29])

	Blood subculture	*In situ* hybridization-based methods	DNA-microarray-based methods	Nucleic-acid amplification-based methods
Duration[a]	Very slow	Fast or very fast	Fast	Fast or very fast
Complexity	Laborious	Laborious	Simple	Simple
Role	Standard diagnostics	On-top assay	On-top assay	On-top assay
Limit of detection	1–10 CFU/mL	10^6 CFU/mL	10^6–10^7 CFU/mL	10^2–10^6 CFU/mL
Suitability for therapy monitoring	Yes (bacteremia; conditionally: time to positivity)	Conditionally (with live/dead staining)	No (only conditionally indirectly via: time to positivity of culture)	No (only conditionally indirectly via: time to positivity of culture)
Costs[b]	<10€ (if negative), 10–50€ (if positive)	10–50€	50–250€	30–150€
Laboratory type (setting)	Satellite	Satellite	Centralized or academic	Satellite or standard (assay-dependent)
Limitations	Very specialized technicians	Very specialized technicians Most methods do not provide information about antimicrobial resistance	Variable sensitivity	False-positive results from artefacts

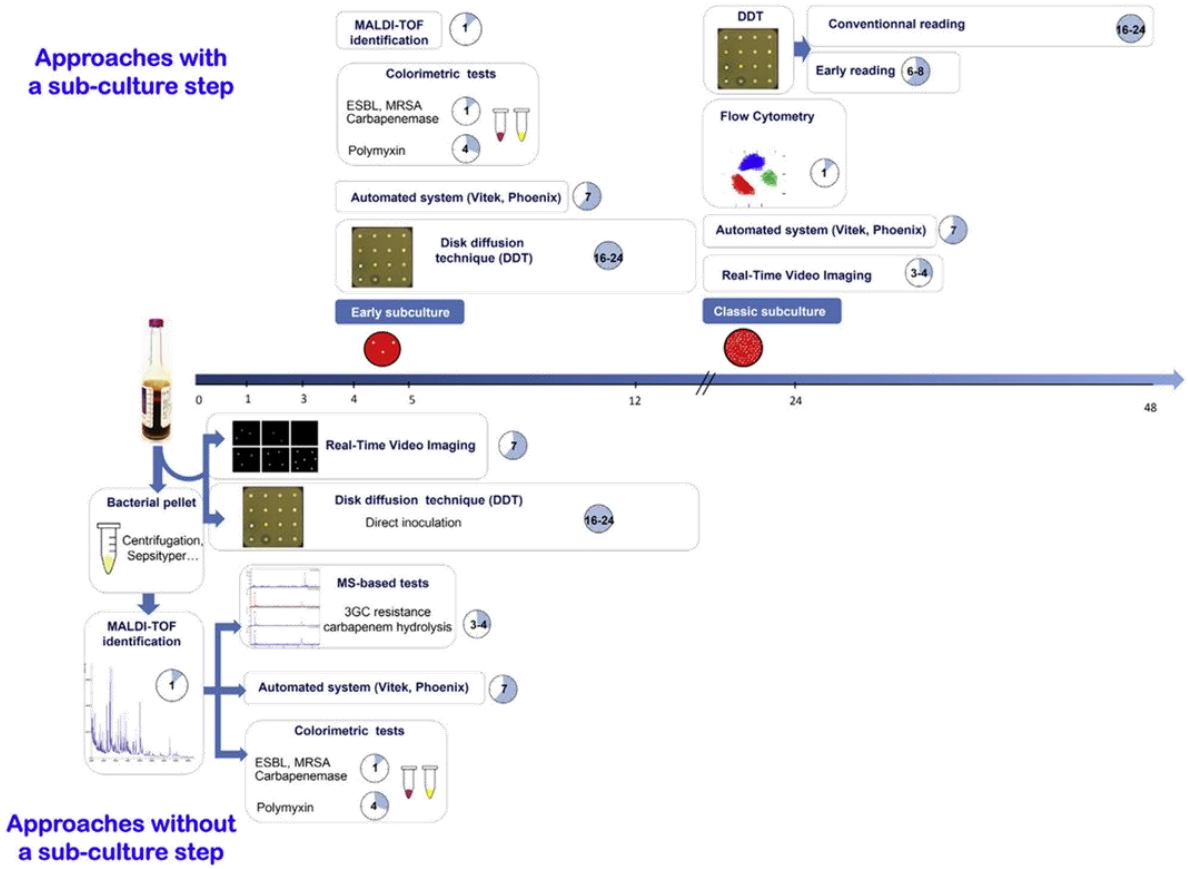
Quantification is not addressed as these assays all start from highly enriched cultures.
[a] Four categories were considered: Very slow (6–48h); Slow (3–6h); Fast (1–3h); Very fast (<1h).

Given all these reasons, molecular techniques and other rapid methods are not considered replacements of the conventional methods[35].

In order to speed-up the microbiological BSI diagnostic process of the phenotypic techniques, many studies have evaluated the application of conventional tests directly from positive BC bottles[37–43]. The major advantage of these applications is the amount of provided information, such as multiple antimicrobials tested with the possibility of minimum inhibitory concentration (MIC) reporting. On the contrary, the major disadvantages of these rapid or clinical AST are: the application only to monomicrobial positive BC, the lack of a standardized 0.5 *McFarland* inoculum, made of viable bacterial cells, with consequent moderate agreement with standard AST, the limited TAT reduction by avoiding the necessity of isolated colonies from overnight subcultures without acting on the test TAT[43,44]. Moreover, the modified and adapted test procedure often needs numerous manual steps, such as lysis and centrifugation steps, with an additional workload on laboratory personnel. Another phenotypic method used to reduce TAT is immunochromatography, that allows to report the presence of enzymes

responsible of resistance to selected antimicrobials categories (*e.g.*, CTX-M, KPC, VIM, etc.) directly from positive BC bottles and in few minutes[45]. As previously stated for molecular methods, the results provided by this technique mainly allow to infer the presumptive identification of ineffective antimicrobials. The main rapid phenotypic methods applied in BSI diagnostic process with relative TAT and starting point are described in Figure 3[44].

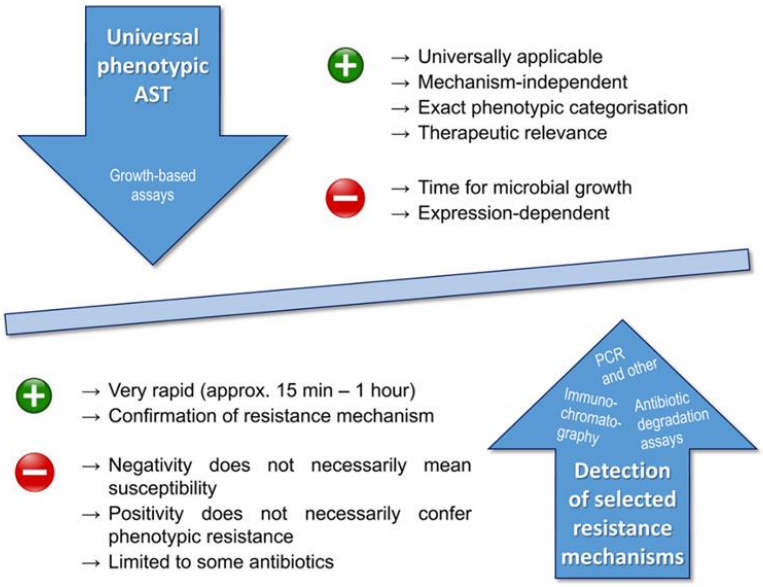
Figure 3 - Main rapid in-house and commercially available phenotypic methods for BSI (from Dubourg et al. 2018)[44]



Interesting results, both in terms of TAT and in terms of produced information, could be reached by the combination of molecular and phenotypic technologies[46–49]. This diagnostic strategy is a good compromise between the strengths of the different diagnostic methods, particularly the timeliness, and the wealth of information regarding the AST. Usually, molecular methods, for example FISH or PCR, are used for microorganisms ID directly from positive blood cultures or from bacterial biomass grown

after a short-term incubation on solid medium and phenotypic methods are used to provide AST results.

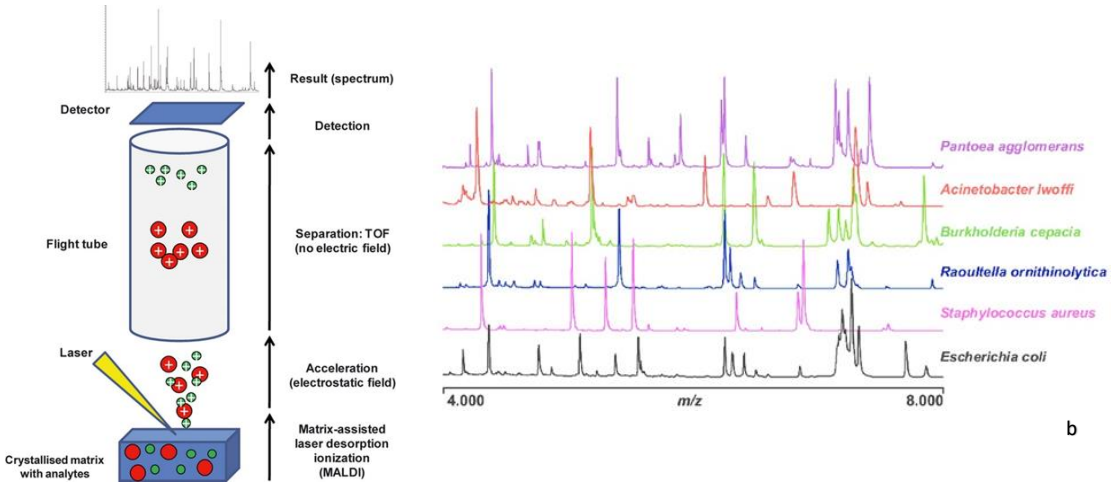
Figure 4 - Phenotypic and genotypic methods pros and cons in rapid AST application (from Idelevich et al. 2019)[32]



Matrix-assisted laser desorption ionization-time of flight mass spectrometry

One of the most widely used molecular method for microorganisms ID in the hub CML, is the Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). This new technology, in contrast with the previously presented genetic methods, is based on proteomic analysis, and can rapidly and accurately identify a wide range of microorganisms with a high level of SE and SP[50].

Figure 5 - MALDI-TOF MS operating principles (a)[51] *and bacterial spectra examples (b)*[52]



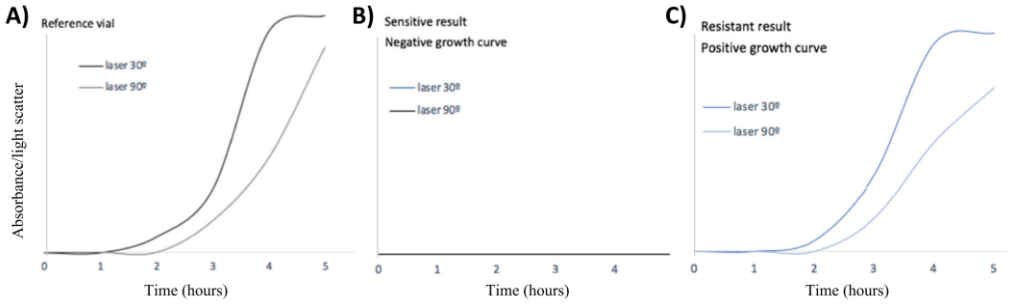
MALDI-TOF MS can generate, directly from the analysis of the mass-to-charge ratio (m/z) of ribosomal proteins, a characteristic wave spectrum of the selected microorganism[50]. The spectrum of the unknown microorganism is compared with a library of thousands of known microorganisms spectra to reach the microbial ID[21,53,54]. By this process unknown microorganisms can be correctly identified. MALDI-TOF MS is able to identify a wide range of different microorganisms belonging to different groups, not only usual bacteria, but also yeasts, moulds, mycobacteria and actinomyces, covering almost the totality of aetiological agents of BSI. Its TAT is of few minutes with a reduced manual operator workload, although isolated colonies usually, obtained by overnight subcultures of biological samples, are needed as starting point. With the adoption of different protocols, the advantages of MALDI-TOF MS can be applied to speed-up the BSI diagnostic workflow and rapidly identify microorganism from positive BC. The protocols are divided in two major groups, those applied directly to positive BC bottles through different manual steps, such as lysis and centrifugation, or those based on a short-term incubation time of positive BC broth on solid medium[54].

Light scattering technology

A new phenotypic method to determine bacterial AST, usually performed in combination with MALDI-TOF MS to reduce BSI TAT, is represented by Light Scattering Technology (LST). It is a versatile system that can be used for different purposes such as the execution of rapid bacterial cultures, the determination of Residual Antimicrobial Activity, the screening for multidrug resistant organisms (MDRO) and for rapid AST. LST is based on liquid based enrichment media and real-time reading system of the light scattered at 30° and 90° reading angles to the incident light, by two distinct detectors. The scattering signals are elaborated and converted into microbial growing curves with a reading sensitivity 100 times higher than the photometric systems. It's application in BSI begins with a small amount of positive BC broth that is subcultured in another growth broth until the achievement of a standard inoculum of alive bacterial cells of 0.4 - 0.6 *McFarland*. At this point the AST can be set-up and the growing curves in the presence of a tested antimicrobial are interpreted by the system into categorical AST

information. AST TAT performed by LST is of 3 - 5 hours after reaching the standard inoculum.

Figure 6 – Example of AST performed by LST (from Anton-Vazquez et al. 2019)[55]

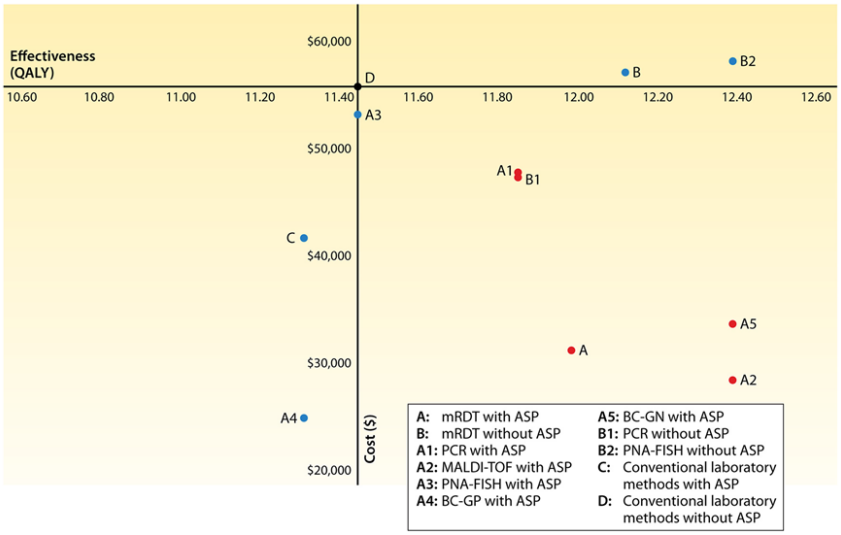


"A) represents the standard growth curve without presence of antibiotic (curves are displayed representing the light scattered signals received by photodetectors placed at 30° and 90° from the laser beam). B) represents a sensitive or susceptible antimicrobial result. C) Represents a resistant result, in the presence of antibiotic the growth curve is positive, comparable to the reference vial"[55]

Bloodstream infections diagnostic workflow and Antimicrobial Stewardship

The panorama of rapid diagnostic tests in Clinical Microbiology is ever expanding. As previously reported, molecular and phenotypic methods, especially if combined, can easily reduce BSI microbiological diagnosis TAT under 8 hours. However, BSI and sepsis burden in terms of morbidity and mortality rates is still growing. Data on the impact of these rapid diagnostic workflows, especially in real-life settings, are scarce, and, for the emerging technologies, sometimes conflicting[56–58]. A systematic review published in 2018 highlighted how the potential and promising capabilities of rapid diagnostic tests could be downsized by different factors such as laboratory opening hours, the lack of rapid communication of the results from the laboratory to the physicians and, most of all, the lack of Antimicrobial Stewardship (AS) programmes[57–59]. CML actively contributes within the AS programmes with different duties[58,60]. One of the most important current challenges for CML is to integrate the new diagnostic technologies in the hospital real-life organization, considering not only the diagnostic tests strengths and weaknesses but also other pivotal factors such as the restricted number of tests that can be performed at the same time per platform, the bounded human and economic resources to allow a wide exploitation of these technologies, and the medical need of timely results in defined critical groups of patients[59,61].

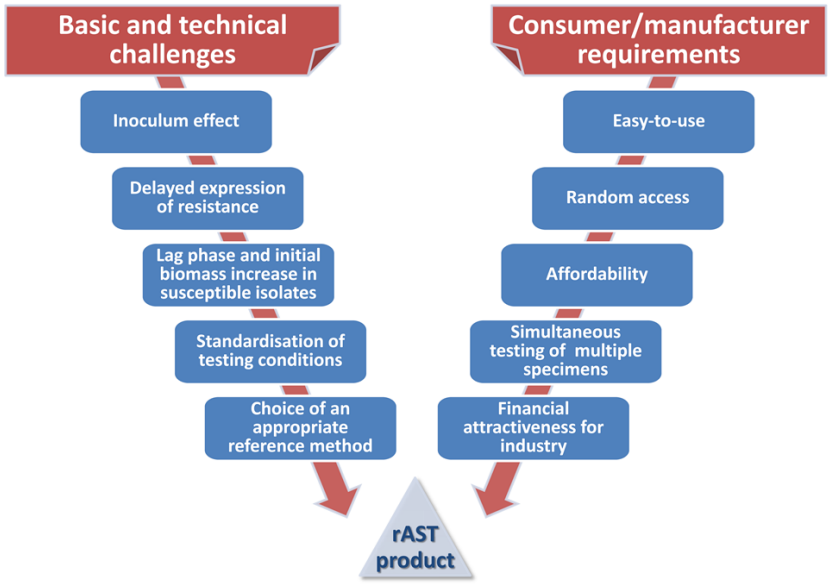
Figure 7 - Cost-effectiveness analysis of different rapid molecular and phenotypic methods for BSI diagnosis (from Pliakos et al. 2018)[57]



mRDT: molecular rapid diagnostic test; PNA-FISH: peptide nucleic acid fluorescent in situ hybridization; BC-GP: blood culture nanotechnology microarray system for Gram-positive bacteria; BC-GN: blood culture nanotechnology microarray system for Gram-negative bacteria; ASP: antimicrobial stewardship programs

This topic is exactly part of the laboratory's tasks within the AS programs: the necessity to set up fast and dedicated diagnostic pathways to promote the allocation of the diagnostic resources to gain the maximum clinical impact for rapid tests[59,61,62].

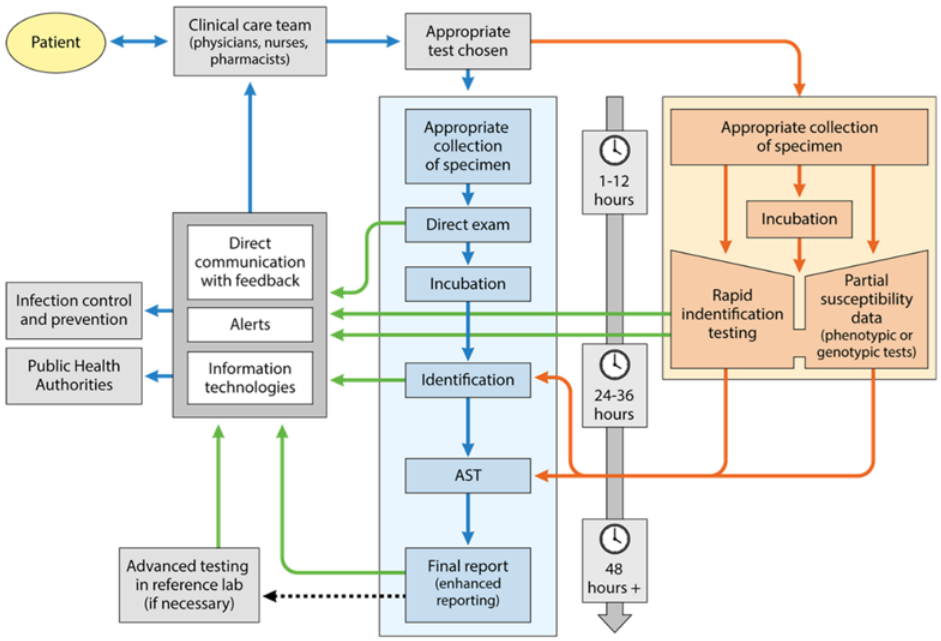
Figure 8 - Tests handicaps and CML needs in the selection process of rapid AST (from Idelevich et al. 2019)[32]



Within AS programmes, infections control is the most effective measure to contrast the spread of antimicrobial resistance phenomenon. Timely microbiological laboratory reports, therefore, contribute to put in place infection control protocols to avoid the

spread of MDRO. Correct antimicrobial prescription policies involve the appropriate use of empirical antimicrobial therapies. In addition to the direct and indirect benefits of promoting rapid diagnostic testing, CML can contribute to AS even before the report of the fastest microbiological test currently available.

Figure 9 - Conventional and rapid microbiological diagnostic workflows, their interconnection points and relationship with AS programmes (from Morency-Potvin et al. 2017)[63]



"Blue arrows represent the conventional microbiology pathway, orange arrows represent the RDT pathway, and green arrows represent opportunities for the laboratory and antimicrobial stewardship teams to improve communication of results. AST, antimicrobial susceptibility testing."[63]

Only a precise knowledge of the local epidemiology and cumulative AST data at hospital level provided by CML can guide targeted-empirical antibiotic treatment approaches, thus promoting a rational use of antibiotics and containing the phenomenon of antimicrobial resistance[60].

Objectives

The aim of the *PhD* project was to design, set-up and evaluate a rapid protocol (RP) for BSI microbiological diagnosis combining the advantages of molecular proteomic methods with the phenotypic ones. For molecular methods MALDI-TOF MS was chosen for the timeliness in ID process directly applied to positive BC and the wide library of identifiable microbial *species*. For phenotypic methods, LST was chosen for the rapidity of the test, the possibility to customize the antimicrobial panels to be tested and the wealth of provided information.

At the *Teaching Hospital Molinette* of the *A.O.U. Città della Salute e della Scienza di Torino*, the *PhD* project was articulated in two interconnected parts. The first consisted in the construction of a dataset of information useful for the set-up of the diagnostic test and for AS policies in selected Internal Medicine, Geriatric and Emergency Medicine wards, setting of application of the rapid diagnostic protocol. The local epidemiology, as regards BSI, and the administration of antimicrobials were evaluated during the period 2016 to 2019. The last year of *PhD* project application, 2020, was not investigated due to COVD-19 pandemic and its impact on hospital activity and organization. Contemporary selection criteria for patients and samples were set-up to access to the rapid diagnostic protocol, configuring a potential parallel diagnostic pathway, a fast-track, for BSI diagnosis.

The second part of the *PhD* project was to evaluate, from a technical point of view, the diagnostic accuracy and the TAT of the combined rapid diagnostic test in comparison to the reference methods in use in our laboratory and secondly to assess, in a real-life setting, its potential impact on the therapeutical management of enrolled septic patients. For this purpose, a survey on potential antimicrobial changes on the basis of RP results was submitted to the treating physicians. The registered answers were compared with the antibiotic regimens that could be administrated based on reference protocol results.

Materials and methods

The study was conducted at the *Teaching Hospital Molinette* of the *A.O.U. Città della Salute e della Scienza di Torino*, Turin, Italy. Eight hospital units with different features and functions were involved. Seven clinical wards were included for epidemiological and antimicrobial administration evaluation and for patients enrolment. The CML performed microbiological testings, data collection and analysis. The selected hospital units and their activity in the study were summarized in Table 7.

Table 7 - Involved Hospital Units

Unit type	Unit name	Study activity
Internal Medicine Ward	*S.C.D.U. Medicina Interna 1*	• Epidemiological and antimicrobial administration evaluation • Patients enrolment and management
	S.C.D.U. Medicina Interna 2	
	S.C.D.U. Medicina Interna 3	
	S.C.D.U. Medicina Interna 4	
	S.C. Medicina Interna 5	
Geriatric Ward	*S.C.D.U. Geriatria e Malattie Metaboliche dell'Osso*	
Emergency Medicine Ward	*S.C.D.U. Medicina Urgenza*	
Clinical Microbiological Laboratory	*S.C. Microbiologia e Virologia U.*	• Microbiological tests • Data collection and analysis

Preliminary evaluation of wards epidemiology and baseline data for rapid protocol set-up and application

To pursue the first part of the *PhD* project, microbiological epidemiology, target population, and setting were evaluated from 2016 to 2019, two years before the potential application of the experimental protocol, and in the following period with the exclusion of 2020. Data collection in 2020, indeed, was not assessed due to COVD-19 pandemic and its impact on hospital activity and organization.

Microbiological epidemiology

A preliminary evaluation of the number of BSI episodes in the selected wards was performed during the period 2016 – 2017. By epidemiological software *Mercurio* -

infection control and awareness v. 1.5 (*Dedalus, Italy*), microorganisms isolated from BC in the selected wards were extracted. Only the first microorganism per patient within 28 days was considered. Data from 2016 to 2017 were analysed to estimate the potential pathogens coverage of the RP and to guide the choice of antimicrobials to be tested: cumulative AST were elaborated for the 3 most frequently detected bacterial *species* with at least 30 isolates for the full considered period[64]. Monitoring of BSI episodes and data analysis was extended to 2018 and 2019 in the same mode.

Antiinfectives for injective use prescription analysis

The prescription trend of ATC class J01, antiinfectives for injective use, was investigated in the selected wards, across the period 2016 – 2019. The quantity of ordered antibiotic packs was extracted by the informatic corporate software *OLIAMM*. The antimicrobial prescription report was expressed in Defined Daily Dose (DDD) over 100 days of hospitalization[65].

Population and setting description

Ward population and setting were described through the information extracted from the Hospital Discharge Register. The number of hospital admissions, patients age, mortality rate, DRG charge were analysed from 2016 to 2019. DRG average standardized charge was calculated for each selected ward as follows: the DRG, coded from the International Classification of Diseases, 9th Revision, were summed per year and divided by the number of admissions in the same year.

Study design and selection of patients and samples

For the second part of the study, the evaluation of a new rapid BSI diagnostic workflow, a monocentric prospective observational study was designed. The study started on July 2018 and the end was foreseen after 2 years.

Patients admitted to the selected hospital wards during the study period satisfying the selection criteria were prospectively enrolled for the study. All the following criteria needed to be present simultaneously:

- adult patients (age ≥18 years);
- patients with sepsis diagnosis;

- patients hospitalized at the time of blood culture positivity in the selected wards: Internal Medicine, Geriatric, Emergency Medicine;
- patients with monomicrobial positive blood culture bottles;
- patients with blood cultures positivity within 10 a.m. from Monday to Thursday;
- written informed consent.

One of the following situations was enough to exclude patients from the study:

- patients with blood cultures positive for anaerobic or fastidious bacteria or yeasts;
- patients with blood cultures positivity from 10:00 to 15:00;
- patients with blood cultures positivity from 10:00 of the penultimate working day to 15:00 of the last non-working day.

For patients enrolment, sepsis was defined according to Sepsis-2 criteria (Table 8): two or more satisfied conditions at the same time as the result of infection[66].

Table 8 - Sepsis-2 definition criteria (from Bone et al. 1992)[66]

SIRS criteria
Two or more of: • Temperature >38°C or <36°C • Heart rate >90/min • Respiratory rate >20/min or $PaCO_2$ <32 mmHg (4.3 kPa) • White blood cell count >12000/mm^3 or <4000/mm^3 or >10% immature bands

The conditions of RP feasibility or not in relationship to BC positivity time and presumed timing for Gram stain, SP and RP ID and AST results, are summarized in Figure 10, Figure 11 and Figure 12.

Figure 10 - Condition for RP application (ideal condition with BC positivity within 10:00 from Monday to Thursday)

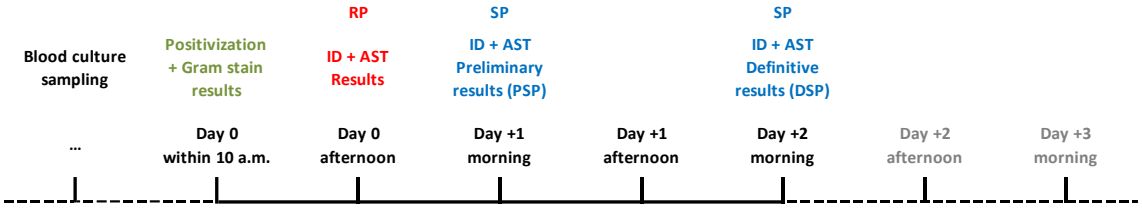


Figure 11 - Condition for RP application (ideal condition with BC positivity after 15:00 from Monday to Thursday)

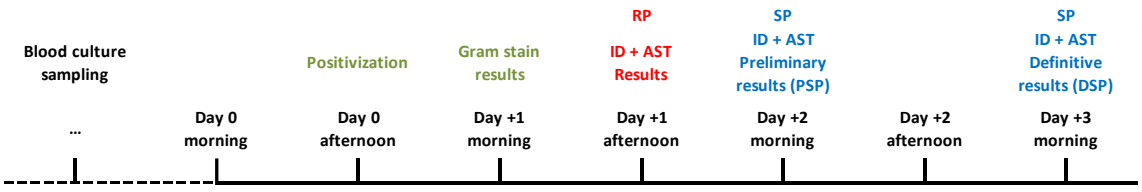
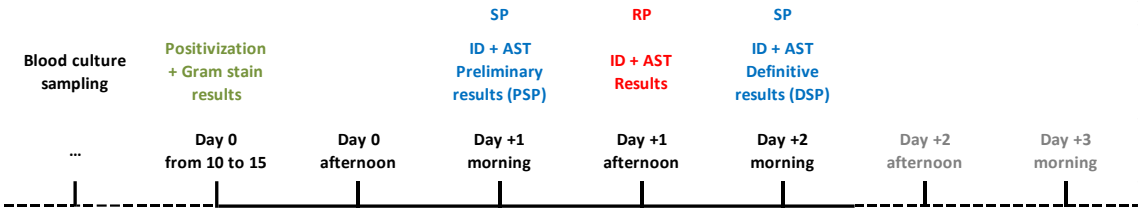


Figure 12 - Condition of non-feasibility of RP (uselessness of RP with BC positivity between 10:00 to 15:00)



During the same hospitalization, only the 1st microorganism grown in the BC sets per septic episode was analysed.

Anamnestic information and clinical-laboratory parameters

For enrolled patients, personal data (sex, date of birth, age), anamnestic information on previous hospitalization, comorbidities, known allergies, antimicrobial therapies in the previous 6 months, clinical and laboratory parameters at BC collection and physicians survey time, were collected.

Diagnostic Protocols

The collected BC bottles during septic episodes were transported to the *S.C. Microbiologia e Virologia U.* and loaded in the *BACT/ALERT VIRTUO* (*bioMérieux, France*) instrument with the manufacturer incubation protocol of 5 days at 35-37°C. BC bottles flagged positive were prospectively considered and Gram staining observation was performed. Selected BC, after the eligibility criteria check, were analysed using both Standard (SP) and Rapid protocols (RP) in parallel.

Standard protocol (SP)

Bacteria ID and AST were simultaneously performed by the phenotypic microdilution-based automated instrument *Microscan Walkaway 96 plus System* (*Beckman Coulter, USA*). *Microscan Walkaway ID/AST* panels *Pos Combo 33* and *Neg BP Combo 46* (*Beckman Coulter, USA*) were used for Gram-positive and Gram-negative bacteria, respectively. In the same day of blood culture positivity (day 0), positive monomicrobial BC bottles were subcultured on appropriate solid media relying on Gram staining results.

Five drops of positive BC were plated on Columbia agar with 5% sheep blood (*Becton–Dickinson, USA*) and spread over the plate surface with a 10 µL inoculation loop. The blood agar plates were incubated at 37°C in 5% CO_2 controlled atmosphere.

PRELIMINARY STANDARD PROTOCOL (PSP)

After 3 to 5 hours incubation time (day 0), from the growing bacterial patina on blood agar plates, a 0.5 *McFarland* bacterial suspension was prepared. The standardized suspension was used to set up *Microscan Walkaway ID/AST* panels, according to manufacturer protocol, and achieve preliminary ID and AST results on the next day (day +1).

DEFINITIVE STANDARD PROTOCOL (DSP)

On the next day (day +1), another ID and AST assay was performed from overnight subcultures with presumed available results on the 2nd day from BC positivity (day +2).

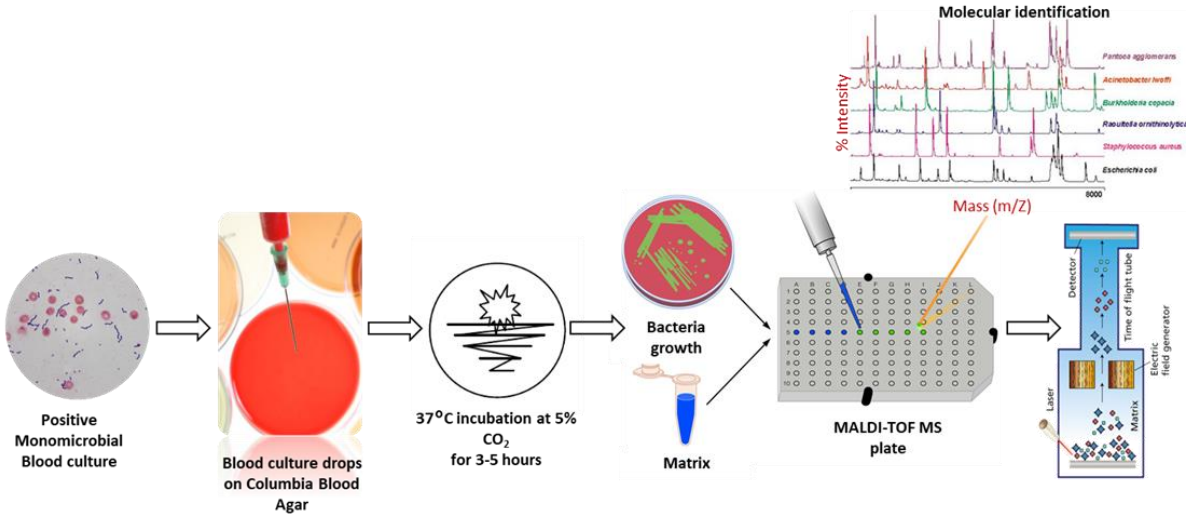
Rapid protocol (RP)

The RP was conducted in parallel to SP.

IDENTIFICATION

After 3 to 5 hours of incubation time, the same blood agar plate adopted in the PSP for preliminary ID and AST, was used for ID by MALDI-TOF MS. Briefly, a thin layer of growing colonies was scraped and spotted on MSP 96 steel targets plate (*Bruker Daltonik GmbH, Germany*), overlaid with 1 µl of α-Cyano-4-hydroxycinnamic acid matrix solution (*Bruker Daltonik GmbH, Germany*) and air dried.

Figure 13 - Rapid protocol: bacterial identification



The target plate was loaded on *Bruker Microflex LT* (*Bruker Daltonik GmbH, Germany)* mass spectrometer for bacterial mass spectra acquisition. The produced spectra were compared with *MALDI Biotyper RUO library v. 8.0* by *MALDI Biotyper software v. 3.1*. (*Bruker Daltonik GmbH, Germany)* to achieve ID. *Bruker Bacterial Test Standard* (*Bruker Daltonik GmbH, Germany*) was used for session calibration according to manufacturer's instructions. Interpretation criteria, as recommended by the manufacturer, are summarized in Table 9. Growing colonies with no reliable identification results were reincubated and reanalysed using the same procedure.

Table 9 - MALDI-TOF MS interpretation criteria

Interpretation	Range	Colour
Species-level identification	2.00 - 3.00	Green
Genus-level identification	1.70 - 1.99	Yellow
No reliable identification	<1.70	Red

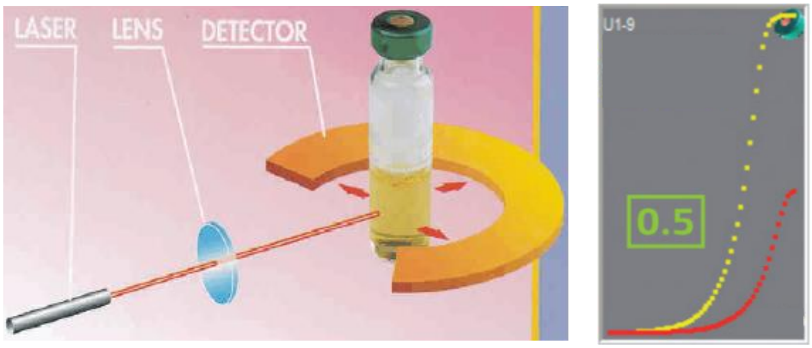
Bacterial identification process is summarized in Figure 13. RP ID results were used to interpret AST performed with LST.

ANTIBIOTIC SUSCEPTIBILITY TESTING

AST was performed from positive BC samples, in parallel to RP ID process, using LST on *ALFRED 60AST* (*Alifax, Italy*) instrument. *ALFRED 60AST* is an automated system with a

60 samples carrousel, a refrigerated store for reconstituted ready-to-use drugs and equipped with a mechanical pipetting arm.

Figure 14 - Light Scattering Technology (LST) operating principle



To perform rapid AST, a standard inoculum of 0.4 - 0.6 *McFarland* from positive blood culture was prepared as follows: an *HB&L* glass vial *(Alifax, Italy)*, containing Brain Heart Infusion enrichment broth, was inoculated with 30µl of the selected positive BC broth and incubated in the instrument at 37°C, in constant mixing by a magnetic stirrer at the bottom of the vial. Scattering signals were detected every 5 minutes at 2 angles with respect to the beam propagation plane at 30° and at 90°. They were elaborated and converted by the instrumental software into growing curves and, through a mathematical elaboration, into *McFarland* and Colony Forming Unit per ml quantitations. After reaching 0.4 - 0.6 *McFarland* turbidity, the standardized bacterial suspension was used to set up the rapid AST. 100 µl of bacterial solution and 200 µl of the selected antibiotic to be tested were automatically transferred by the 3 axes dispensing arm of the instrument to pre-loaded AST glass vials, Automation kit vials (*Alifax, Italy*). As reported in Figure 15, for every analysed sample, an AST vial without antibiotic, was used as reference to interpret the growing curves in the AST vials with antibiotics. AST interpretation was automatically performed by instrument's software and the results were expressed in percentage of resistance to the antibiotic and classified as resistant (R), intermediate (I) or sensitive (S) according to EUCAST Clinical Breakpoint of the current year[67,68].

Figure 15 - Rapid AST interpretation

A customized panel of antibiotics was chosen on the basis of Gram stain results. In Table 10 and Table 11, are reported the antibiotic panels divided by Gram stain features with related TAT for single antibiotic.

Table 10 - Panel of antibiotics available for Gram-positive bacteria

Gram-positive	Antibiotic panel	Time (hours)
Enterococci	Ampicillin	3
	Vancomycin	5
Staphylococci	Cefoxitin	3
	Co-trimoxazole	3
	Daptomycin	3
	Vancomycin	5

Table 11 - Panel of antibiotics available for Gram-negative bacteria

Gram-negative	Antibiotic panel	Time (hours)
Enterobacteriaceae and *Pseudomonas spp.*	Piperacillin-tazobactam	5
	Ceftazidime	5
	Ciprofloxacin	3
	Colistin	3
	Meropenem	5

The antibiotic panels were chosen according to local microbiological epidemiology and empiric antimicrobial therapy protocols in use the selected wards and reported on the

"*Manuale di Terapia Antibiotica Empirica - Reparti di Medicina, Chirurgia Generale e Urologia*"[69].

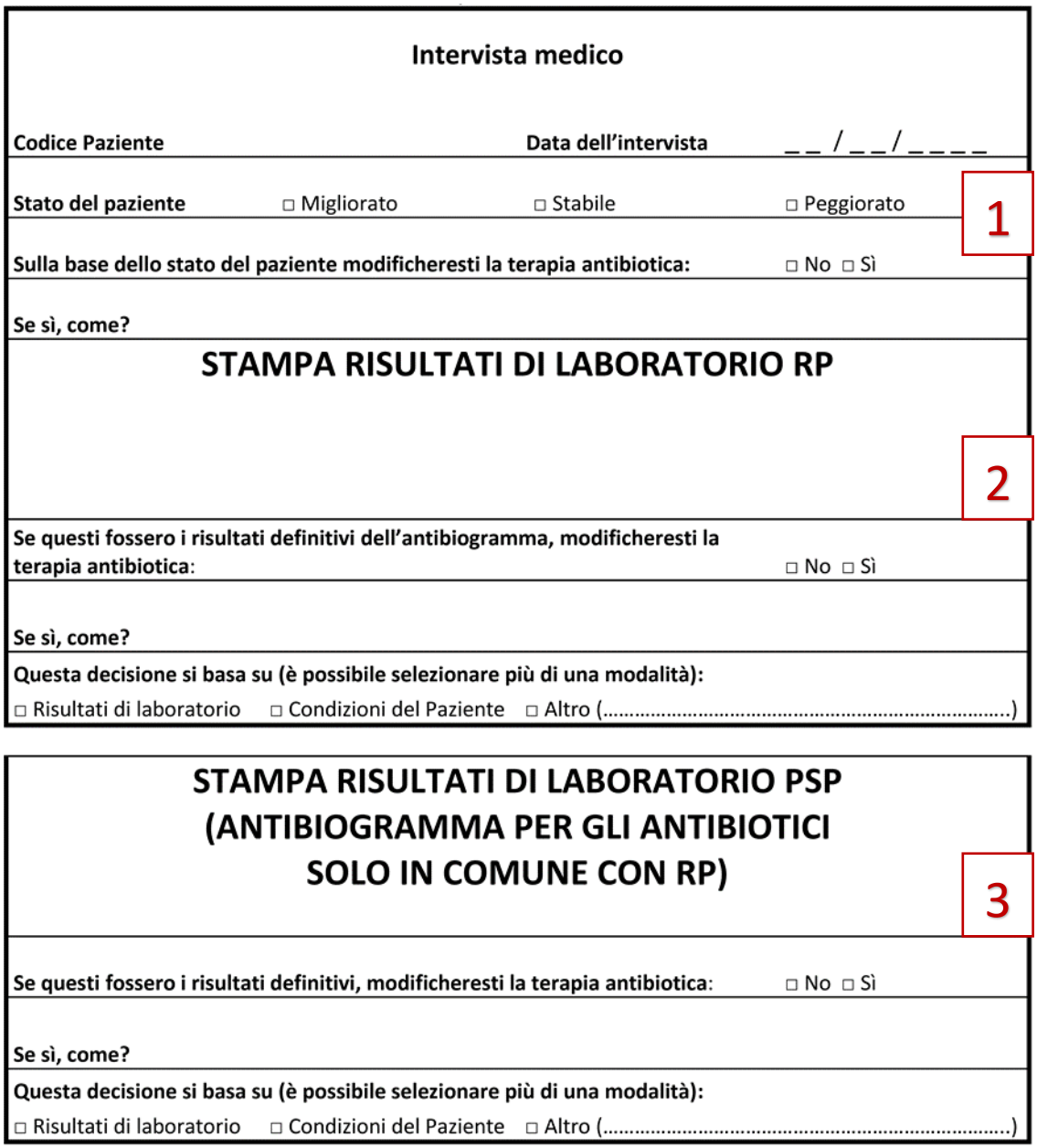
When 0.4 – 0.6 *McFarland* turbidity was reached, *ALFRED 60AST* automatically set-up the AST pre-set by the operator on the basis on Gram staining results. EUCAST lyophilised antibiotics (*Alifax, Italy*) were previously manually dissolved in 2 ml of regenerating solution (*Alifax, Italy*), and loaded in the instrumental cooling unit and stored at 4°C for up to 5 days except for meropenem (3 days).

Antimicrobial therapy management and survey

SP and RP AST results were used to interview physicians on potential antimicrobial therapy changes. The same day of PSP results, before microbiological report consulting, a clinical microbiologist performed a survey on the potential treatment decisions on the basis of patient clinical status, experimental RP and PSP results. Potential treatment choices were registered in 4 different steps. Firstly, clinical status and experimental RP results influence were recorded, then PSP findings. These data were divided in two different sections, first, PSP information were masked for MIC value and additional antibiotics in comparison with RP (defined Short Panel PSP), then complete PSP results were shown for the survey.

Declared antibiotic treatment changes were registered step by step into a dedicated form (Figure 16 and Figure 17). After physician survey, antimicrobial therapy modifications were recorded up to 10 days after DSP results were available. For data analysis only declared choices were considered.

Figure 16 - Survey on potential antibiotic treatment decisions (1/2)



Intervista medico

Codice Paziente **Data dell'intervista** _ _ / _ _ / _ _ _ _

Stato del paziente □ Migliorato □ Stabile □ Peggiorato

1

Sulla base dello stato del paziente modificheresti la terapia antibiotica: □ No □ Sì

Se sì, come?

STAMPA RISULTATI DI LABORATORIO RP

2

Se questi fossero i risultati definitivi dell'antibiogramma, modificheresti la terapia antibiotica: □ No □ Sì

Se sì, come?

Questa decisione si basa su (è possibile selezionare più di una modalità):

□ Risultati di laboratorio □ Condizioni del Paziente □ Altro (.....)

STAMPA RISULTATI DI LABORATORIO PSP (ANTIBIOGRAMMA PER GLI ANTIBIOTICI SOLO IN COMUNE CON RP)

3

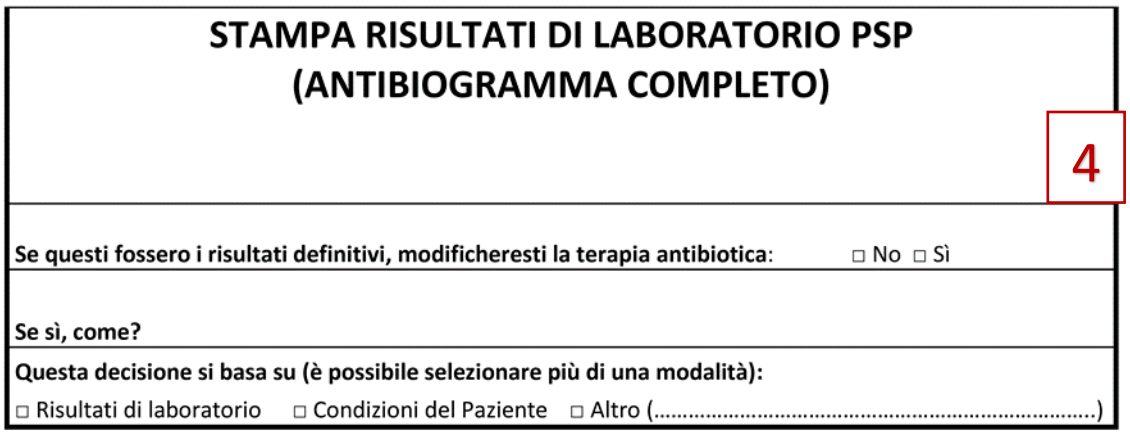
Se questi fossero i risultati definitivi, modificheresti la terapia antibiotica: □ No □ Sì

Se sì, come?

Questa decisione si basa su (è possibile selezionare più di una modalità):

□ Risultati di laboratorio □ Condizioni del Paziente □ Altro (.....)

Figure 17 - Survey on potential antibiotic treatment decisions (2/2)



STAMPA RISULTATI DI LABORATORIO PSP
(ANTIBIOGRAMMA COMPLETO)

4

Se questi fossero i risultati definitivi, modificheresti la terapia antibiotica: ☐ No ☐ Sì

Se sì, come?

Questa decisione si basa su (è possibile selezionare più di una modalità):

☐ Risultati di laboratorio ☐ Condizioni del Paziente ☐ Altro (.....)

Statistical tests

Patients characteristics were summarized using absolute and relative frequency or using percentile (p) according to the distribution. In order to describe BSI in the selected wards, absolute and relative frequency were reported, for each year over 2016 – 2019. Resistance prevalence of the most frequent microorganisms to the RP tested antibiotics were compared to the resistance prevalence in enrolled patients in the observational study.

RP identification results performed were compared with conventional methods and reported as percentage of concordance.

Verification of RP AST results was done in comparison to conventional methods used in SP. Categorical agreement was analysed according to ISO 20776-2 criteria (Table 12)[70].

Table 12 - Categorical agreement according to ISO 20776-2 criteria[70]

		Reference method		
	AST Interpretation	**S**	**I**	**R**
Tested method	**S**	None	Minor error	Very major error
	I	Minor error	None	Minor error
	R	Major error	Minor error	None

AST agreement and errors percentages of acceptability are summarized in Table 13[70].

Table 13 - Categorical agreement: percentages of acceptability[70]

	Acceptability
Categorical agreement	≥ 90%
Minor errors	≤ 10%
Major errors	≤ 3%
Very major errors	≤ 1.5%

Concordance between RP and SP results was assessed using Cohen's kappa coefficient and the strength of agreement was evaluated using threshold reported in Table 14[71].

Table 14 - Cohen's kappa coefficient and strength of agreement[71]

Cohen's kappa coefficient	**Strength of agreement**
<0.00	*Poor*
0.00 - 0.20	*Slight*
0.21 - 0.40	*Fair*
0.41 - 0.60	*Moderate*
0.61 - 0.80	*Substantial*
0.81 - 1.00	*Almost perfect*

Boxplots of TAT of different protocols were reported in order to describe the distribution.

The agreement between different protocols-based decisions was estimated as the proportion of same decisions out of the total evaluable decisions. Moreover, according to the study protocol 90% confidence intervals were reported. As sensitivity analysis, agreement between different protocols-based decisions (and relative 90% confidence interval) in subgroup was assessed.

Research ethics approval

The study was reviewed and approved by the Hospital Ethics Review Committee. Patients received information sheets, the physicians discussed the study with them in light of the details provided in the information sheets and obtained written consent from patients willing to participate. Enrolled patients did not undergo any additional sampling, followed the normal diagnostic-therapeutic pathway based on conventional results, so that decisions regarding antibiotic treatment were based on usual clinical

practice and were not influenced by RP results. RP data were used only for the administration of the survey.

Results

Microbiological epidemiology

At the *S.C. Microbiologia e Virologia U.* of the *Teaching Hospital Molinette* of the *A.O.U. Città della Salute e della Scienza di Torino*, considering the selected wards, more than 400 BSI episodes on at least 6500 ward admissions per year were registered during the period 2016 – 2019. The punctual data divided by year are reported in Table 15 and Table 16.

Table 15 - Isolated microorganisms from blood cultures in 2016 and 2017

2016	N.	%	2017	N.	%
Staphylococcus epidermidis	89	21.5	*Staphylococcus epidermidis*	110	25.1
Escherichia coli	53	12.8	*Escherichia coli*	64	14.6
Staphylococcus aureus	51	12.3	*Staphylococcus aureus*	48	10.9
Staphylococcus haemolyticus	35	8.5	*Staphylococcus hominis*	37	8.4
Staphylococcus hominis	21	5.1	*Staphylococcus haemolyticus*	23	5.2
Enterococcus faecalis / faecium	13 / 18	3.1 / 4.3	*CoNS (others)**	20	4.6
Klebsiella pneumoniae	17	4.1	*Enterococcus faecalis / faecium*	19 / 15	4.3 / 3.4
Candida albicans / non albicans	16 / 16	3.9 / 3.9	*Klebsiella pneumoniae*	17	3.9
*CoNS (others)**	14	3.4	*Viridans group streptococci*	14	3.2
Pseudomonas aeruginosa	13	3.1	*Candida albicans / non albicans*	12 / 5	2.7 / 1.1
Enterobacter species	9	2.2	*Enterobacter species*	11	2.5
Viridans group streptococci	9	2.2	*Bacteroides species*	5	1.1
Bacteroides species	4	1.0	*Proteus mirabilis*	5	1.1
Proteus mirabilis	4	1.0	*Pseudomonas aeruginosa*	5	1.1
Acinetobacter baumannii	3	0.7	*Acinetobacter baumannii*	3	0.7
Bacillus species	3	0.7	*Clostridium species*	3	0.7
Streptococcus groups A, B, C and G	3	0.7	*Enterococcus species (others)*	3	0.7
Morganella morganii	2	0.5	*Kocuria and Micrococcus species*	2	0.5
Clostridium species	2	0.5	*Serratia species*	2	0.5
Citrobacter species	2	0.5	*Stenotrophomonas maltophilia*	2	0.5
Achromobacter xylosoxidans	2	0.5	*Streptococcus pneumoniae*	1	0.2
Kocuria and Micrococcus species	2	0.5	*Other species (only 1 isolated)*	13	3.4
Streptococcus pneumoniae	1	0.2			
Other species (only 1 isolated)	12	3.1			
Total	**414**	**100.0**	***Total***	**439**	**100.0**

*CoNS: *Coagulase Negative Staphylococci*

Table 16 - Isolated microorganisms from blood cultures in 2018 and 2019

2018	N.	%	2019	N.	%
Staphylococcus epidermidis	90	19.5	*Staphylococcus epidermidis*	105	22.5
Escherichia coli	77	16.7	*Escherichia coli*	65	13.9
Staphylococcus aureus	51	11.0	*Staphylococcus aureus*	62	13.3
Staphylococcus hominis	40	8.7	*Staphylococcus hominis*	53	11.4
Enterococcus faecalis / faecium	24 / 14	5.2 / 3.0	*Klebsiella pneumoniae*	24	5.2
Klebsiella pneumoniae	24	5.2	*Staphylococcus haemolyticus*	21	4.5
*CoNS (others)**	23	5.0	*CoNS (others)**	15	3.2
Staphylococcus haemolyticus	20	4.3	*Pseudomonas aeruginosa*	14	3.0
Pseudomonas aeruginosa	15	3.2	*Candida albicans / non albicans*	12 / 14	2.6 / 3.0
Candida albicans / non albicans	14 / 8	3.0 / 1.7	*Viridans group streptococci*	12	2.6
Viridans group streptococci	10	2.2	*Enterococcus faecalis / faecium*	8 / 9	1.7 / 1.9
Corynebacterium species	6	1.3	*Bacteroides species*	7	1.5
Enterobacter species	6	1.3	*Enterobacter species*	5	1.1
Acinetobacter baumannii	4	0.9	*Proteus mirabilis*	5	1.1
Citrobacter species	4	0.9	*Streptococcus groups A, B, C and G*	4	0.9
Serratia species	4	0.9	*Acinetobacter baumannii*	3	0.6
Proteus mirabilis	3	0.6	*Citrobacter species*	3	0.6
Streptococcus groups A, B, C and G	3	0.6	*Serratia species*	3	0.6
Enterococcus species (others)	2	0.4	*Actinomyces species*	2	0.4
Klebsiella oxytoca	2	0.4	*Campylobacter species*	2	0.4
Stenotrophomonas maltophilia	2	0.4	*Clostridium species*	2	0.4
Streptococcus pneumoniae	2	0.4	*Corynebacterium species*	2	0.4
Other species (only 1 isolated)	14	3.2	*Klebsiella oxytoca*	2	0.4
			Streptococcus pneumoniae	2	0.4
			Other species (only 1 isolated)	10	2.4
Total	**462**	**100.0**	***Total***	**466**	**100.0**

*CoNS: *Coagulase Negative Staphylococci*

The principal bacteria involved in BSI were constantly represented by Coagulase Negative Staphylococci (37.4-43.3%), *Enterobacterales* (21.7-26.4%), in particular *Escherichia coli* (12.8-16.7%) and *Staphylococcus aureus* (10.9-13.3%). In the preliminary evaluation of RP application, RP potential pathogens coverage was of the 83.3 and 87.0% in 2016 and 2017, respectively. Considering bacteria, the coverage rose over 90% and even more by selecting only non-fastidious bacteria. In the subsequent years, the

percentages have been approximately preserved. RP coverage data are summarized in Table 17.

Table 17 - Percentage of RP coverage considering the isolated microorganisms

RP coverage (%)	2016	2017	2018	2019
All microorganisms	83.3	87.0	87.0	85.4
Bacteria	90.3	90.7	91.6	90.5
Non-fastidious bacteria	97.7	98.7	98.3	99.0

A cumulative AST profile was elaborated for *Staphylococcus epidermidis*, *Escherichia coli* and *Staphylococcus aureus*, the 3 most frequently isolated bacteria from 2016 to 2017 and the only 3 bacteria with more than 30 isolates over the entire period 2016 – 2019. Their resistance trend to RP tested antibiotics was analysed too. In the graphics below, the continuous red line shows the percentage of resistant isolates tested with RP and the dashed red lines represent the 90% CI. In the 4 considered years, the most frequently isolated bacterium was *Staphylococcus epidermidis*. Its epidemiological AST profile was the worst in comparison with the other frequently isolated bacteria. The resistance trend to RP tested antibiotics is reported in Figure 18 and Figure 19. The percentage of methicillin-resistant (MR) isolates was over the 80% with a steady trend, instead the resistance to vancomycin was low with no more than 1%. Linezolid, daptomycin and fosfomycin were the only drugs with a constant <20% of resistance. The second microorganism was *Escherichia coli* and its cumulative AST, and the resistance trend are described in Figure 20 and Figure 21. The antibiotics with a constant <20% of resistance were piperacillin/tazobactam, meropenem, amikacin, colistin and fosfomycin. As regards 3rd generation cephalosporins, ceftazidime had the lowest percentages of resistance between 27% and 35%. *Escherichia coli* registered a peak of resistance in 2018 to ciprofloxacin returned in range in 2019. The third most frequently isolated microorganism was *Staphylococcus aureus,* its cumulative AST and the resistance trend are reported in Figure 22 and Figure 23, respectively. Vancomycin, linezolid, daptomycin, fosfomycin and co-trimoxazole maintained a percentage of sensitivity

>80%. MR *Staphylococcus aureus* in 2016 were the 61% but in 2019 the percentage decreased to 33%.

Figure 18 - Staphylococcus epidermidis cumulative AST (2016 - 2019) with antibiotics percentages of resistance

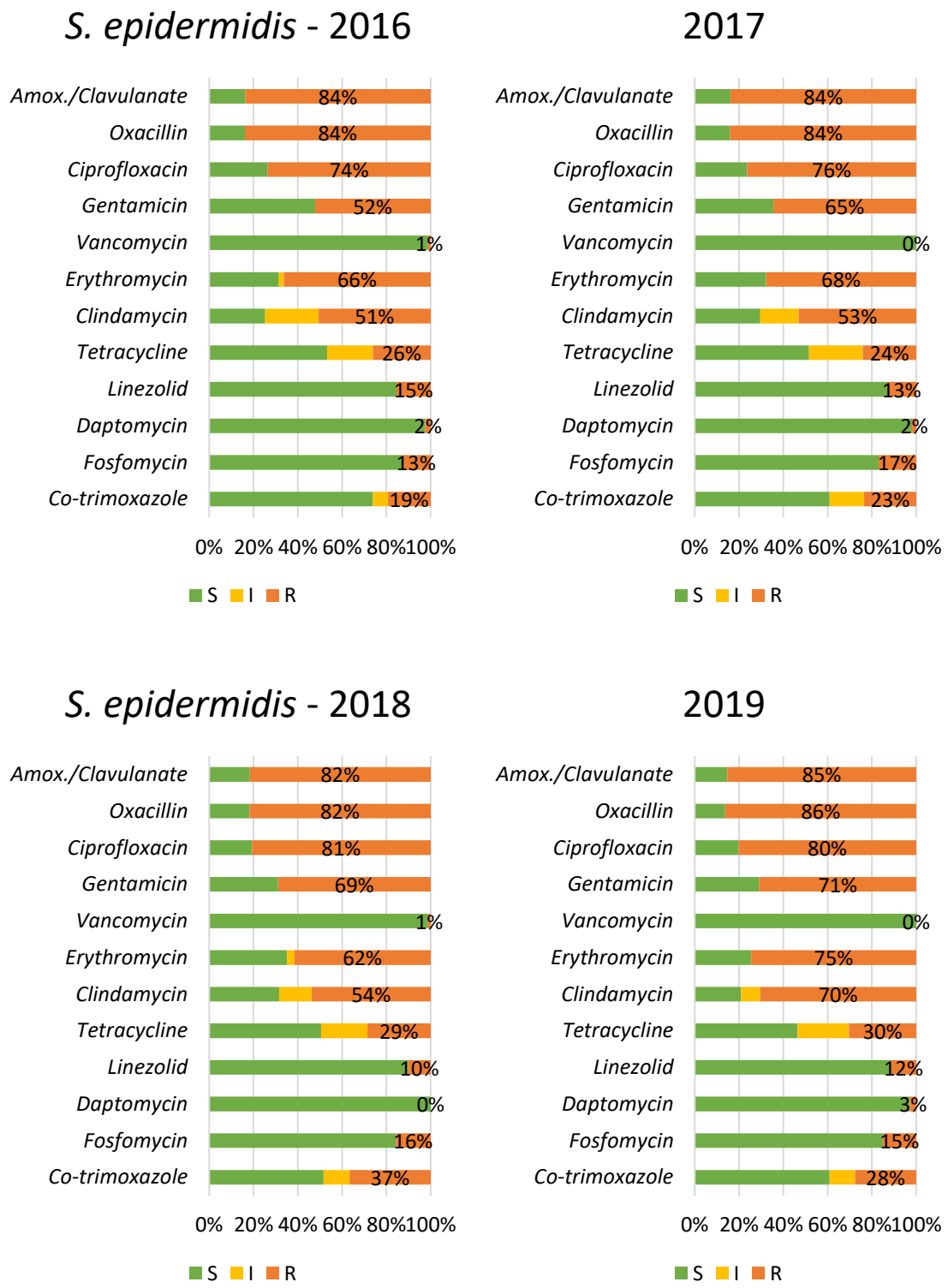
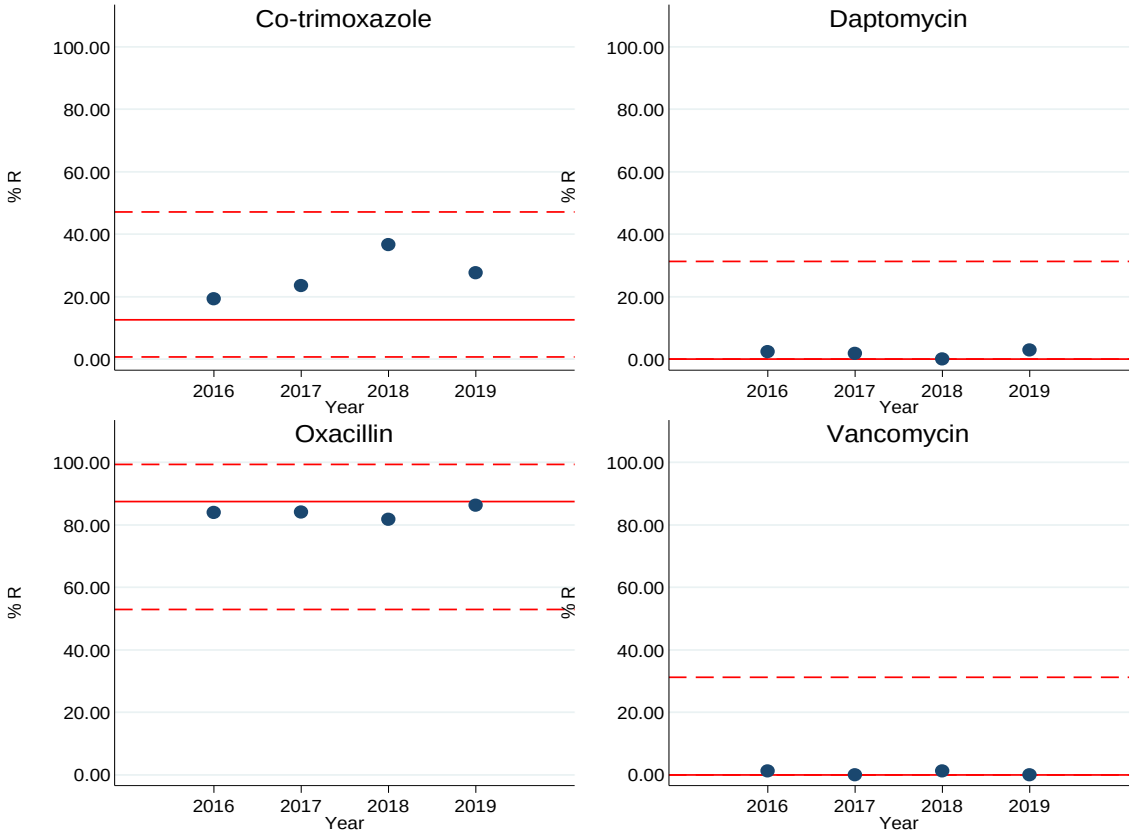


Figure 19 - Resistance prevalence to the RP tested antibiotics of Staphylococcus epidermidis isolated from BSI in selected wards from 2016 to 2019 and resistance prevalence and relative 90% CI in enrolled patients



The continuous red line shows the percentage of resistant isolates tested with RP, the dashed red lines represent the 90% CI, and the blue dots represent the percentage of resistant isolates for each year

Figure 20 - Escherichia coli cumulative AST (2016 - 2019) with antibiotics percentages of resistance

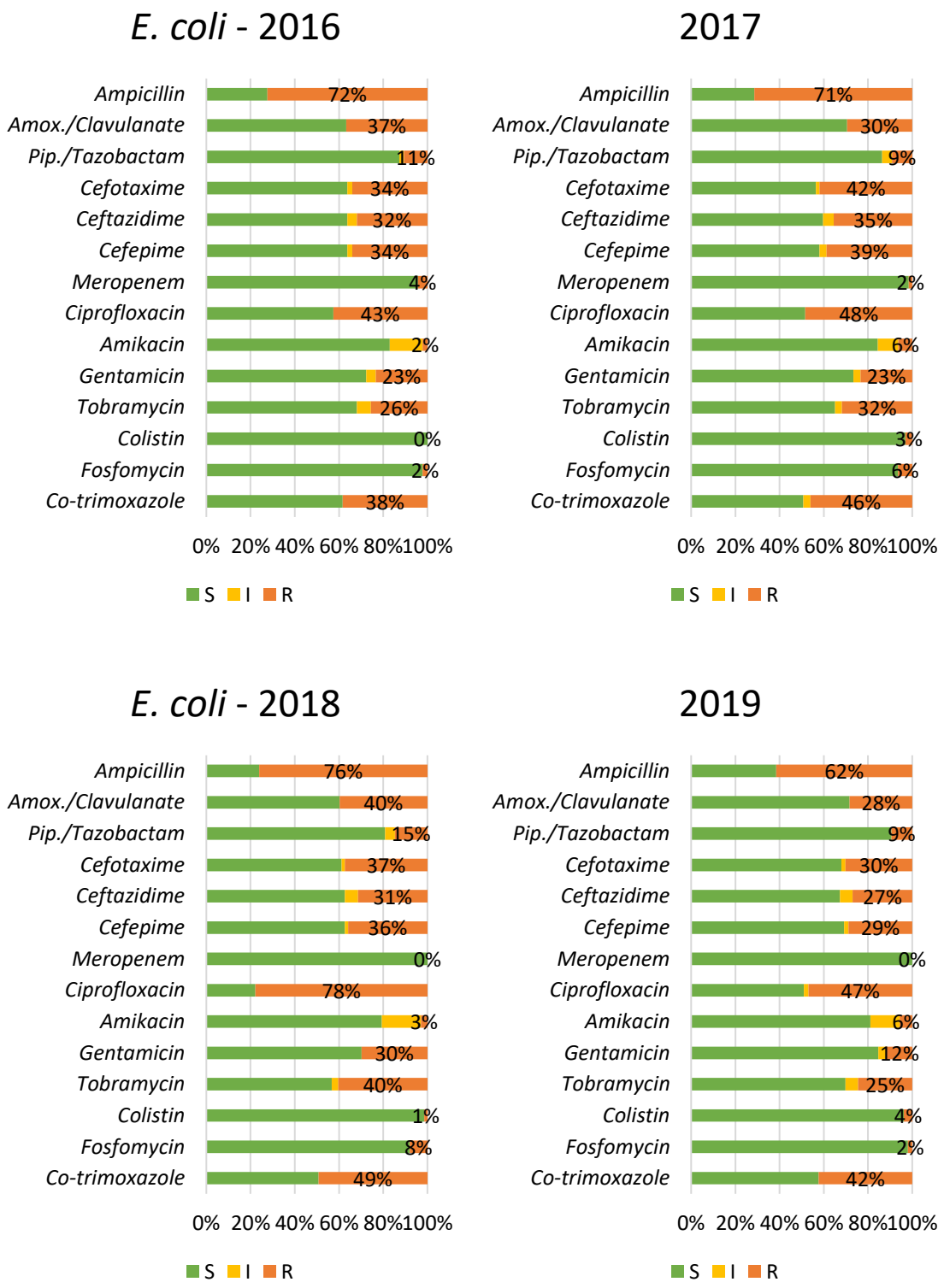
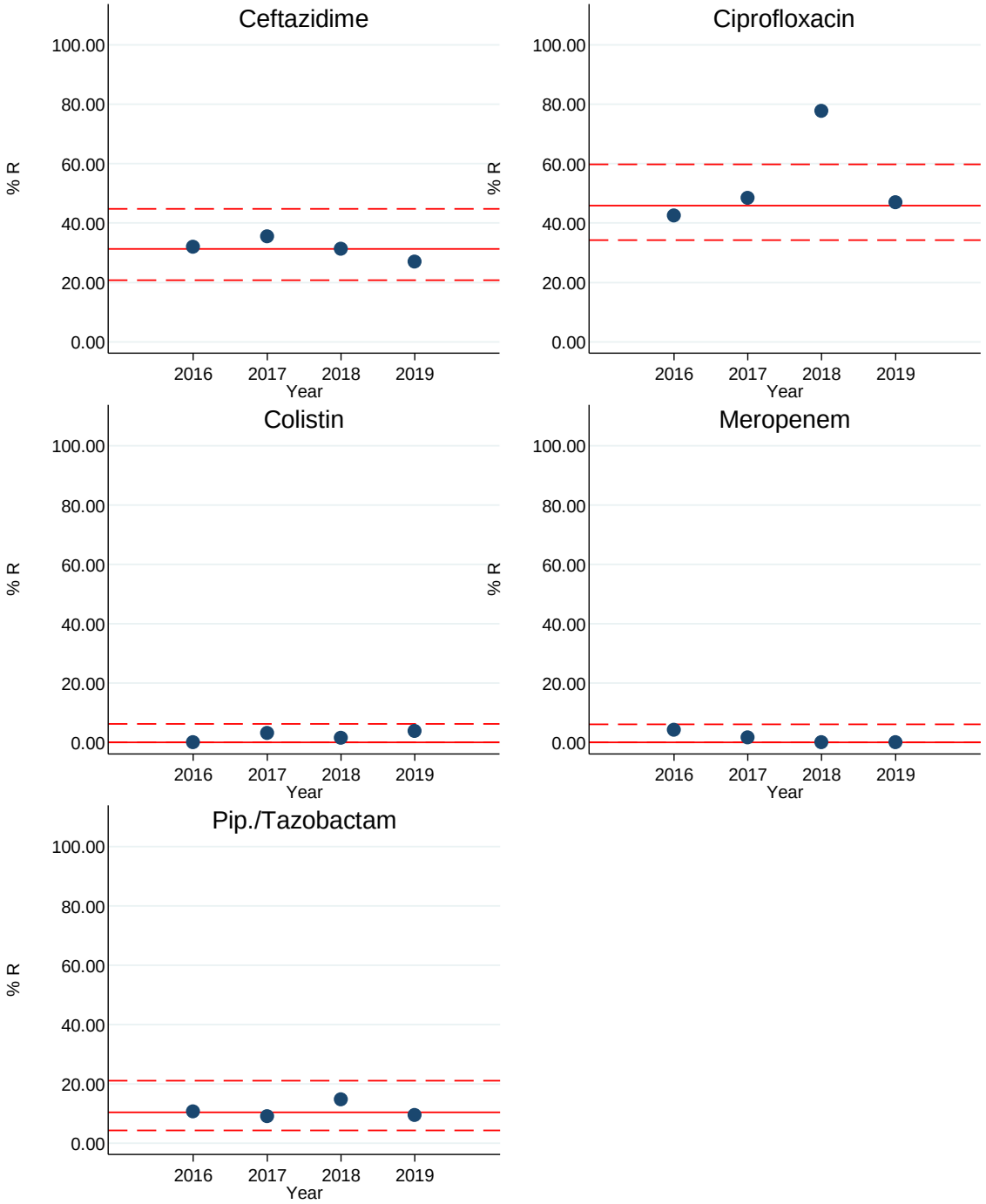


Figure 21 - Resistance prevalence to the RP tested antibiotics of Escherichia coli isolated from BSI in selected wards from 2016 to 2019 and resistance prevalence and relative 90% CI in enrolled patients



The continuous red line shows the percentage of resistant isolates tested with RP, the dashed red lines represent the 90% CI, and the blue dots represent the percentage of resistant isolates for each year

Figure 22 - Staphylococcus aureus cumulative AST (2016 - 2019) with antibiotics percentages of resistance

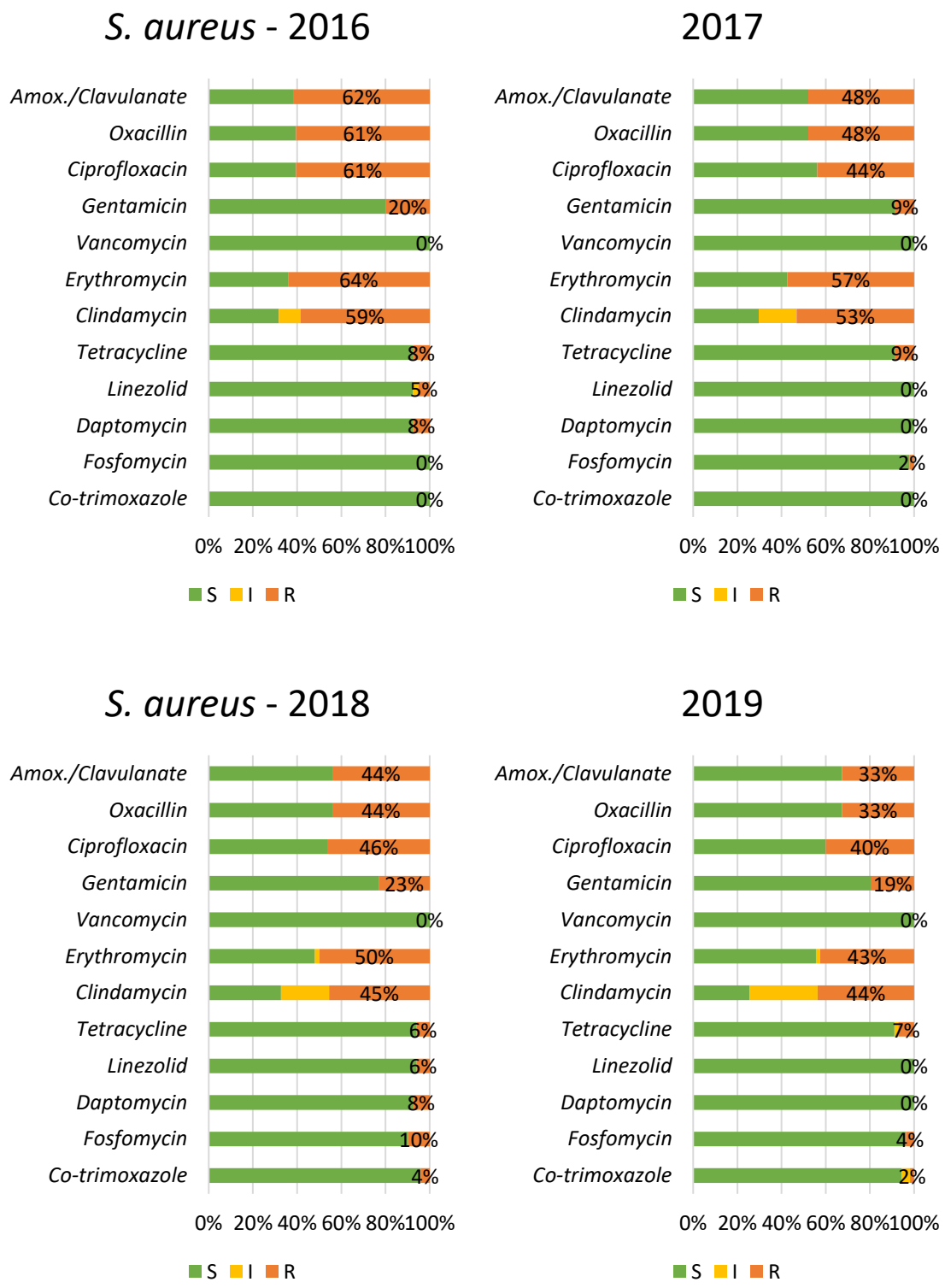
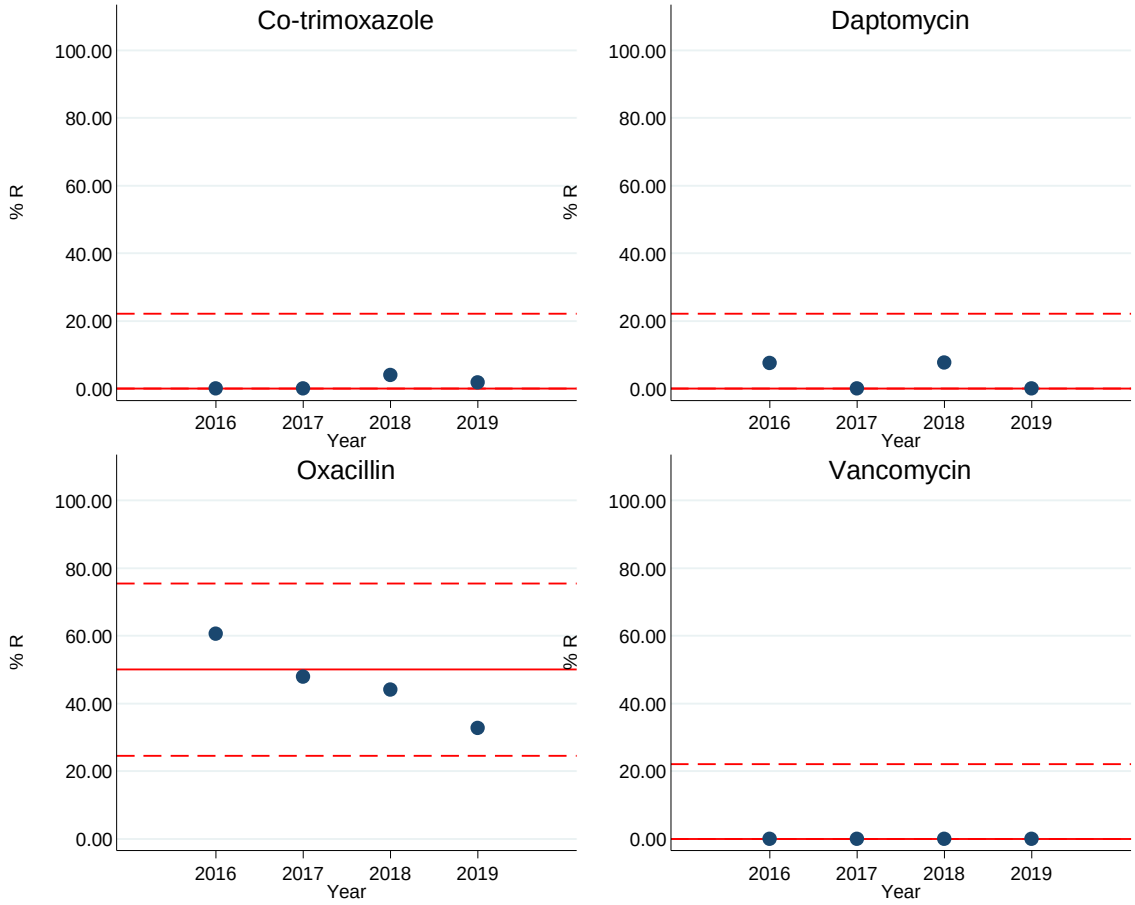


Figure 23 - Resistance prevalence to the RP tested antibiotics of Staphylococcus aureus isolated from BSI in selected wards from 2016 to 2019 and resistance prevalence and relative 90% CI in enrolled patients



The continuous red line shows the percentage of resistant isolates tested with RP, the dashed red lines represent the 90% CI, and the blue dots represent the percentage of resistant isolates for each year

Resistance prevalence did not change in the considered interval time and for all of the RP tested antibiotics was included in the confidence interval (CI) of resistance prevalence in enrolled patients with the exception of Escherichia coli ciprofloxacin resistance in 2018.

Antiinfectives for systemic use prescription analysis

The quantitation of the overall antibiotic consumption of the antiinfectives included in the ATC class J01 during the considered years is reported in Table 18. Beta-lactams were the most used drugs and the group *J01CR – Combinations of penicillins, incl. beta-lactamase inhibitors* was the most prescribed with the exception of the year 2018, where *J01DD – Third generation cephalosporins* passed from the second to the first most administrated antiinfectives. Considering 2017, 2018 and 2019, carbapenems, glycopeptides and quinolones represented the third, fourth and fifth most

administrated classes, respectively. Quinolones consumption trend was progressively in decrease with a more than halved consumption from 2016 to 2019.

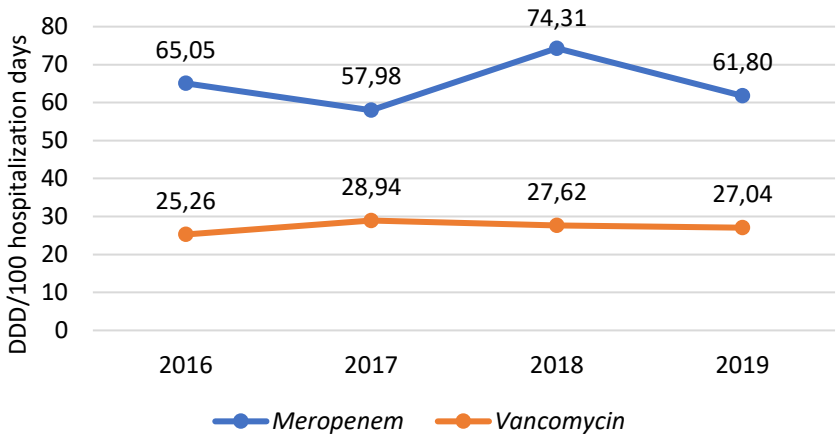
Table 18 - ATC class J01 (antiinfectives for systemic use) prescriptions during 2016-2019

ATC groups of antiinfectives for injective use	2016	2017	2018	2019
J01AA - Tetracyclines	2.24	5.13	4.36	2.31
J01CA - Penicillins with extended spectrum	2.97	0.00	3.51	8.02
J01CE - Beta-lactamase sensitive penicillins	0.00	0.00	0.00	0.00
J01CF - Beta-lactamase resistant penicillins	1.20	1.68	1.40	0.73
J01CR - Combinations of penicillins, incl. beta-lactamase inhibitors	144.03	138.35	99.95	142.77
J01DB - First generation cephalosporins	0.71	0.72	1.43	1.89
J01DC - Second generation cephalosporins	0.00	0.00	0.00	0.00
J01DD - Third generation cephalosporins	98.76	101.32	131.75	127.25
J01DE - Fourth generation cephalosporins	5.01	7.49	31.10	10.61
J01DH - Carbapenems	71.44	62.43	78.44	66.82
J01DI - Other cephalosporins and penems	0.81	2.50	3.14	2.99
J01EE - Combinations of sulfonamides and trimethoprim, incl. derivates	2.97	3.69	2.25	3.88
J01FA - Macrolides	9.80	5.44	10.61	12.77
J01FF - Lincosamides	0.50	0.81	0.46	1.53
J01GB - Other aminoglycosides	8.76	9.10	6.83	5.36
J01MA - Quinolone antibacterials	32.64	31.45	26.03	13.43
J01XA - Glycopeptide antibacterials	28.68	31.80	29.85	28.54
J01XB - Polymyxins	0.64	0.98	1.67	1.43
J01XD - Imidazole derivates	11.90	10.23	15.89	12.11
J01XE - Nitrofuran derivates	0.12	0.00	0.33	0.00
J01XX - Other antibacterials	19.45	18.90	28.07	32.66

From 2016 to 2019, meropenem represented the most prescribed carbapenem with a range from 91.0% to 94.7%. Vancomycin, similarly, with a range from 88.1 to 94.8% was the most administrated glycopeptide. Meropenem and vancomycin prescription trends are reported in Figure 24. Data are expressed over time as DDD over 100 hospitalization days. Vancomycin administration trend was stable both considered all selected wards

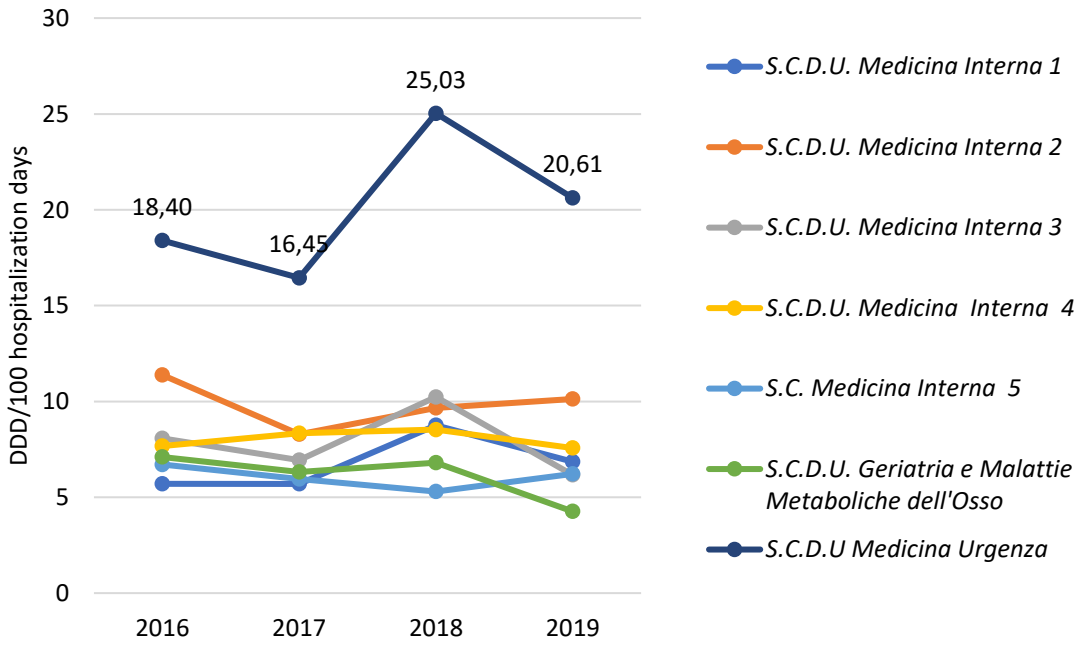
and each ward singularly (data shown in Appendix). As regards meropenem, a peak of prescription was recorded in 2018. Meropenem administration trend, divided for the considered wards, is described in Figure 25.

Figure 24 - Meropenem and vancomycin administration in selected wards (data expresses as DDD/100 hospitalization days)



Considering all selected wards, the augmented meropenem DDD over 100 hospitalization days in the 2018 reflects the increased consumption observed in the Emergency Medicine ward in the same year. As shown in the Figure 25 in the other wards meropenem administration did not change over time.

Figure 25 - Meropenem administration in the selected wards over 2016 - 2019



Target population and wards setting

During the considered years, the number of admissions, the patients age, the mortality rate and the DRG average standardized charge for each ward were stable (Table 19 and Table 20). Data on population age over years and on DRG average standardized charge are detailed in Appendix.

Table 19 - Hospital admissions, age and mortality trends in the selected wards during the period 2016 - 2019

	2016		2017		2018		2019	
	N.	**%**	**N.**	**%**	**N.**	**%**	**N.**	**%**
Hospital admissions	7108		6969		7034		7052	
Median age	78	(14 - 106)	78	(14 - 107)	78	(15 - 103)	78	(14 - 104)
Mortality	577	8.1	664	9.5	669	9.5	682	9.7

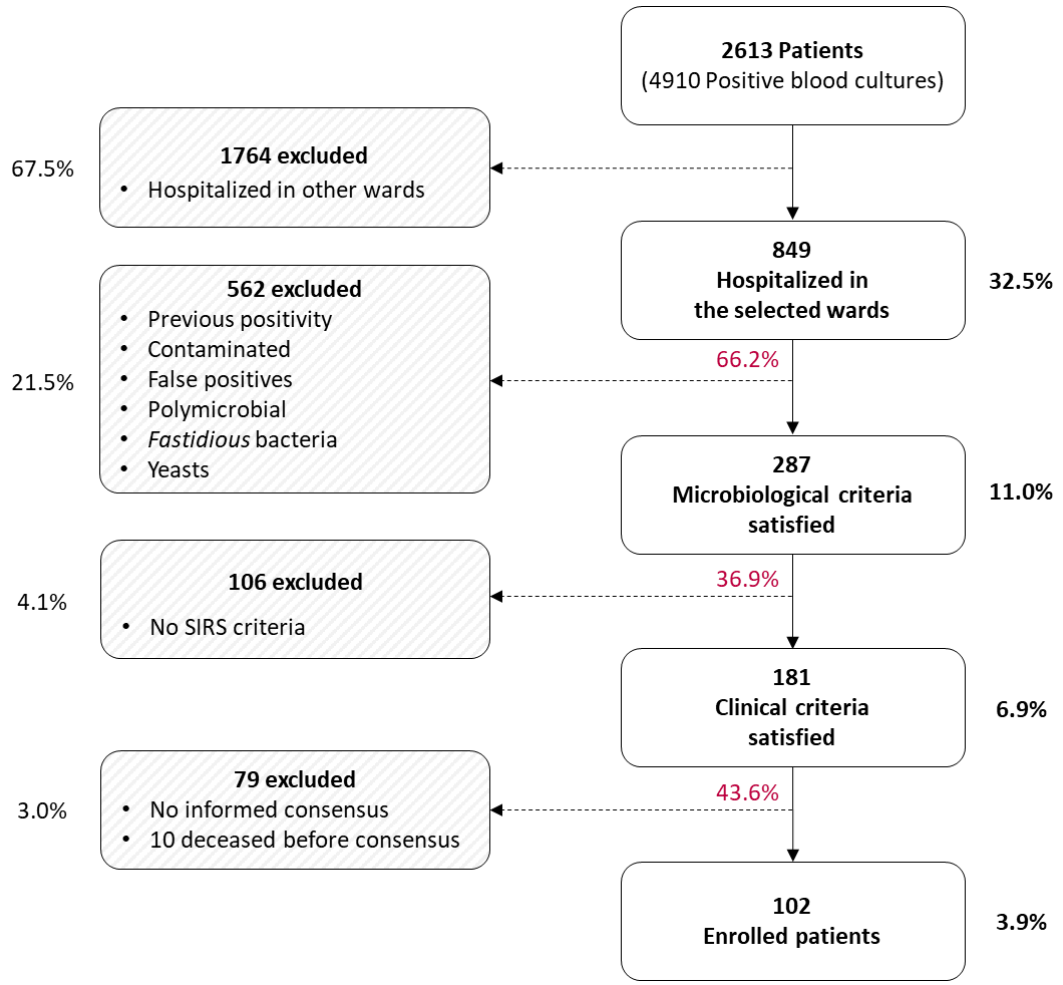
Table 20 - DRG average standardized charge trend divided for selected wards and year

DRG average standardized charge	2016	2017	2018	2019
S.C.D.U. Medicina Interna 1	0.95	0.98	0.93	0.97
S.C.D.U. Medicina Interna 2	1.06	1.08	1.06	1.10
S.C.D.U. Medicina Interna 3	0.94	0.99	0.97	1.01
S.C.D.U. Medicina Interna 4	1.05	1.01	1.02	1.00
S.C. Medicina Interna 5	1.00	1.01	1.01	0.99
S.C.D.U. Geriatria e Malattie Metaboliche dell'Osso	0.94	0.88	0.92	0.88
S.C.D.U. Medicina Urgenza	1.22	1.20	1.40	1.33
Total	**1.14**	**1.19**	**1.16**	**1.17**

At the *S.C. Microbiologia e Virologia U.* of the *Teaching Hospital Molinette* of the *A.O.U. Città della Salute e della Scienza di Torino*, from the 1st July 2018 to the 30th June 2020, 2613 patients with 4910 positive BC in 329 working days were analysed. Of these, 1764 (67.5%) were not hospitalized in the selected wards and were excluded. Of the remaining 849 patients with BSI episodes, 562 (66.2%) did not satisfy the microbiological inclusion criteria: 267 (47.5%) had previous positive BC sets during the same hospitalization, 136 (24.2%) had positive contaminated BC, 57 (10.1%) had polymicrobial BSI, 46 (8.2%) had BSI caused by fastidious bacteria, 35 (6.2%) had fungemia and 21 (3.7%) had false positive blood culture bottles. Of the 287 remaining patients, 106

(36.9%) did not satisfy the SIRS criteria. Seventy-nine (43.6%) patients were subsequently excluded, no written informed consent could be expresses. Finally, 102 patients were considered for the study. A flowchart of the selection process is summarized in Figure 26.

Figure 26 - Patients selection process flowchart



Patients features

Demographic and clinical features are reported in Table 21. One hundred and two septic episodes were considered (47 males and 55 female). Median age of enrolled patients was 78.5 (range 25th- 75th 66.0 - 83.4) and >90 years patients were 5 (4.9%). Over the 50% (53) of patients were overweighted or obese. 81.4% (83) of patients had at least one comorbidity and 45.1% (46) patients had 3 or more. Congestive heart failure, COPD and diabetes were the most frequent diseases.

Table 21 – Patients characteristics at hospital admission

Characteristics	N.	%
Sex		
Female	47	46.1
Male	55	53.9
Age		
≤60	15	14.7
60-69	16	15.7
70-79	27	26.5
80-89	39	38.2
≥90	5	4.9
Body Mass Index		
Underweight (<18.50)	7	6.9
Normal weight (18.50-24.99)	42	41.2
Overweight (25.00-29.99)	42	41.2
Obese (≥30)	11	10.8
Charlson Comorbidity Index		
0	17	16.7
1	27	20.6
2	9	8.8
3	26	25.5
≥4	29	28.4

Comorbidities	N.	%
Myocardial infarction	13	12.7
Congestive heart failure	31	30.4
Peripheral vascular disease	13	12.7
CVA or TIA	13	12.7
Dementia	13	12.7
Hemiplegia	1	1
COPD	29	28.4
Peptic ulcer disease	8	7.8
Kidney failure	6	5.9
Transplanted	6	5.9
HSCT	1	16.7
Kidney	2	33.3
Liver	2	33.3
Kidney-pancreas	1	16.7
Immunodepression	17	16.7
Neoplasm	15	14.7
Localized	6	5.9
Metastatic	9	8.8
Oncohematological disease	11	10.8
Autoimmune disease	10	9.8
Diabetes	28	27.5
Yes, without damage organ	12	11.8
Yes, with damage organ	16	15.7
Liver disease	14	13.7
Mild	10	9.8
Moderate to severe	4	3.9
Allergy	38	37.3
Other diseases	44	43.1

The 40.2% of the patients received an antibiotic regimen in the last 6 months and the 51.0% in the last 30 days before hospital admission. The majority of patients (79.4%) were admitted from Emergency Room for suspected infections.

Table 22 - Patients characteristics before enrolment

	N.	%
Previous antibiotic therapies		
within the last 30 days	41	40.2
within the last 6 months	52	51.0
Previous hospital admissions	48	47.1
within previous 6 months	48	47.1
in Medical ward	38	79.2
in Surgical ward	7	14.6
in Intensive care unit	1	2.1
in more than 1 ward	2	4.2

Table 23 - Patients characteristics at BC sampling

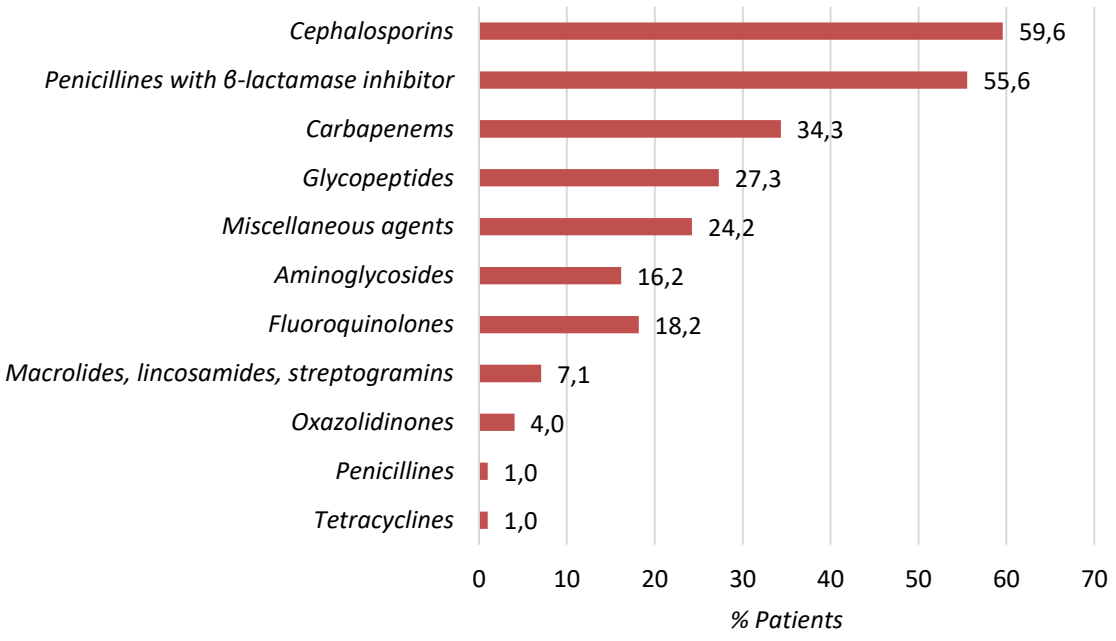
Sepsis diagnostic criteria	**N.**	**%**
Positive SIRS score criteria		
2	73	71.6
3	26	25.5
4	3	2.9
Temperature >38 °C or <36°C	88	86.3
Heart rate >90/min	69	67.4
Respiratory rate >20 breaths/min	20	19.6
WBC >12000/mm^3 or <4000/mm^3 or >10% immature bands	63	61.8
Positive SOFA score criteria		
Not determinable	58	56.7
1	30	29.4
2	13	12.7
3	1	1.0
Systolic pressure ≤100 mmHg	22	21.6
Glasgow come scale ≤14	18	17.6
Respiratory rate ≥22 breaths/min	19	18.6

Most of the enrolled patients (71.6%) had only 2 positive SIRS criteria at the time of septic episode. In 56.7% of the patients, qSOFA score was not determinable due to the lack of at least one parameter. In 49 (48%) patients the respiratory rate was not reported in medical records while pulse oximetry data were present in the 100% of the patients.

Prescribed antimicrobials

The prescribed classes of antibiotics in the selected patients during septic episodes, are reported in Figure 27. Cephalosporins were the most administrated drugs with 46.5% of enrolled patients treated with ceftriaxone. However, piperacillin/tazobactam was the most frequently prescribed antimicrobial (47.5%). Among carbapenems, meropenem and among glycopeptides, vancomycin were the most used with 25.3% and 27.3%, respectively.

Figure 27 - Antimicrobials administrations in enrolled patients



Blood cultures report

For each septic episode, a BC bottle was analysed for a total of 102. Of these the 54.9% were drawn in the Emergency department. The average number of collected BC set was of 2 (range 1-4) with a 90.2% of peripheral venepuncture samplings. The most frequently

isolated bacteria were Gram-negative with 61.8%. The principal features of BC bottles with the isolated microorganisms are summarized in Table 24.

Table 24 - Blood culture bottle features with isolated microorganisms

Blood culture bottles	N.	%
Emergency department	56	54.9
Medicine wards	46	45.1
Peripheral sampling	92	90.2
CVC sampling	10	9.8
Aerobic	53	52.0
Anaerobic	49	48.0
Gram-positive	39	38.2
Gram-negative	63	61.8

Microorganisms	N.	%
Escherichia coli	48	47.1
Staphylococcus aureus	12	11.8
Staphylococcus hominis	11	10.8
Staphylococcus epidermidis	8	7.8
Klebsiella pneumoniae	7	6.9
Enterococcus faecium	3	2.9
Enterobacter aerogenes	2	2.0
Enterococcus faecalis	2	2.0
Klebsiella oxytoca	2	2.0
Citrobacter koseri	1	(1.0)
Enterobacter cloacae	1	1.0
Enterococcus gallinarum	1	1.0
Proteus mirabilis	1	1.0
Staphylococcus capitis	1	1.0
Staphylococcus lugdunensis	1	1.0
Serratia marcescens	1	1.0

Of the 21 CoNS, 81% were MR, as regard the 12 isolated *Staphylococcus aureus*, the 50%. Between the 63 detected *Enterobacterales*, 33.3% were extended-spectrum beta-lactamases-producers (ESBL) and only 1 (1.7%) carbapenemase-producer (CPE). The immunochromatographic characterization revealed the production of a VIM enzyme in a *Klebsiella pneumoniae*. One (16.7%) vancomycin-resistant *Enterococcus* (VRE) *faecium* on 6 was registered.

Figure 28 - Escherichia coli cumulative AST with percentages of antibiotic-resistance

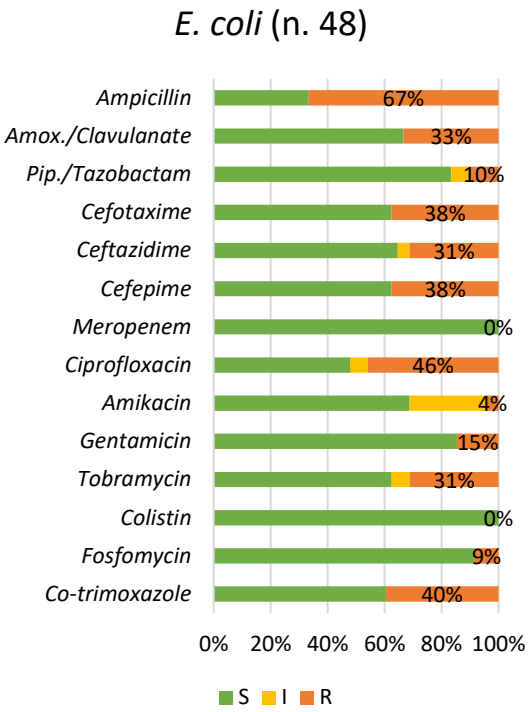


Figure 29 - Staphylococcus aureus cumulative AST with percentages of antibiotic-resistance

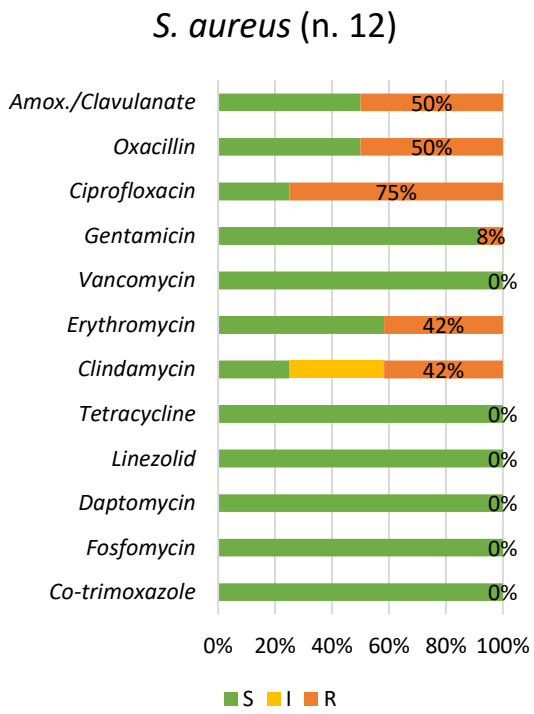
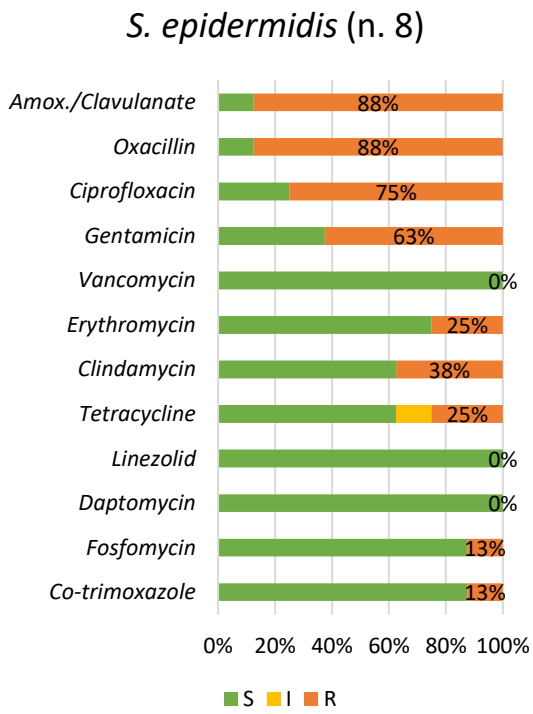


Figure 30 - Staphylococcus epidermidis cumulative AST with percentages of antibiotic-resistance



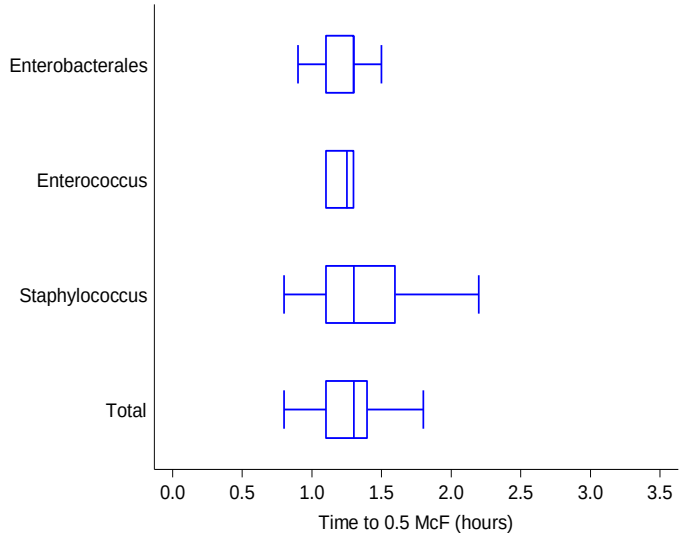
The cumulative AST of *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* are reported in Figure 28, Figure 29 and Figure 30, respectively. The only reliable cumulative AST (>30 determinations) belonged to *Escherichia coli* with 48 determinations.

Time to reach 0.4 - 0.6 *McFarland* with ALFRED60AST system, in general and divided by microbial order or *genus*, is reported in Table 25 and represented graphically in Figure 31.

Table 25 - RP 0.4 - 0.6 McFarland turn-around time expressed in hours

Bacteria	N.	Min	Max	p5	p25	p50	p75	p95	mean	St.dev.
Enterobacterales	63	0.9	2	0.9	1.1	1.3	1.3	1.7	1.28	0.22
Enterococcus	6	0.8	1.3	0.8	1.1	1.25	1.3	1.3	1.17	0.20
Staphylococcus	33	0.8	3.1	0.8	1.1	1.3	1.6	2.9	1.40	0.54
Total	**102**	**0.8**	**3.1**	**0.9**	**1.1**	**1.3**	**1.4**	**1.9**	**1.31**	**0.36**

Figure 31 – Boxplot RP 0.4 - 0.6 McFarland turn-around time



Rapid protocol and Standard protocols results

In 3 cases (2.9%) PSP results were not available due to 1 technical problem and 2 failed growths controls in *Microscan Walkaway ID/AST* panels. For these reasons, data

regarding RP performance were compared with only 99 (97.1%) corresponding PSP results. On the contrary, all the DSP and RP were available for study aims and RP performance evaluation.

Identification results

RP, in comparison with conventional phenotypic methods used in the DSP, achieved a correct microorganisms ID in 100% and 97.1% of the cases at *genus* and *species*-levels, respectively. Three (2.9%) CoNS were not identified at *species*-level. PSP ID performance were lower than RP both at *genus* (97.1%) and *species*-level (94.1%). However, PSP in 3 cases misidentified the *species* of CoNS. Moreover, as previously reported, PSP in 3 (2.9%) cases could not provide ID results: the excluded cases from RP comparison with PSP were all ESBL-producers *Escherichia coli*.

RP and PSP identification performance in comparison with DSP are reported in Table 26.

Table 26 - RP and PSP identification performance in comparison with DSP

	Identification results			
Protocol	***Genus*-level**	***Species*-level**	**Mismatch**	**No reliable/no ID**
Rapid (RP)	102 (100%)	99 (97.1%)	0 (0%)	0 (0%)
Preliminary Standard (PSP)	99 (97.1%)	96 (94.1%)	3 (2.9%)	3 (2.9%)

MALDI-TOF MS provided an ID at least at *genus*-level for all the positive BC samples, and the results were available in the 100% of the cases for AST interpretation.

Antimicrobial susceptibility testing results

The RP was successfully performed for all the 102 microorganisms causing BSI. With 459 AST determinations, the overall agreement of RP in comparison with PSP was of 93.5% with a Cohen's kappa coefficient of 0.81 (95% CI 0.74 - 0.87). Even if the overall RP agreement was almost perfect, the VME and ME percentages exceeded the acceptability range (VME 3.9 *vs.* ≤1.5%; ME 3.9 *vs.* ≤3%). The detailed AST RP agreements and related categorical errors in comparison to PSP are summarized in Table 27 and Figure 32.

Table 27 - RP vs. PSP AST agreement and related categorical errors

Antibiotic	Test	% Agreement	VME	ME	miE	% VME	% ME	% miE
Ampicillin	6	100	0	0	0	0	0	0
Cefoxitin	33	93.9	1	1	0	4.5	9.1	0
Ceftazidime	60	93.3	0	1	3	0	2.4	5
Ciprofloxacin	60	95	0	1	2	0	2.8	3.3
Colistin	60	96.7	2	0	0	50	0	0
Co-trimoxazole	33	81.8	0	1	5	0	3.6	15.2
Daptomycin	33	100	0	0	0	0	0	0
Meropenem	60	100	0	0	0	0	0	0
Piperacillin/tazobactam	60	80	0	10	2	0	18.5	3.3
Vancomycin	39	100	0	0	0	0	0	0
Total	**444**	**93.5**	**3**	**14**	**12**	**3.9**	**3.9**	**2.7**

VME: very major error; ME: major error; miE: minor error

Figure 32 - RP vs. PSP AST agreement

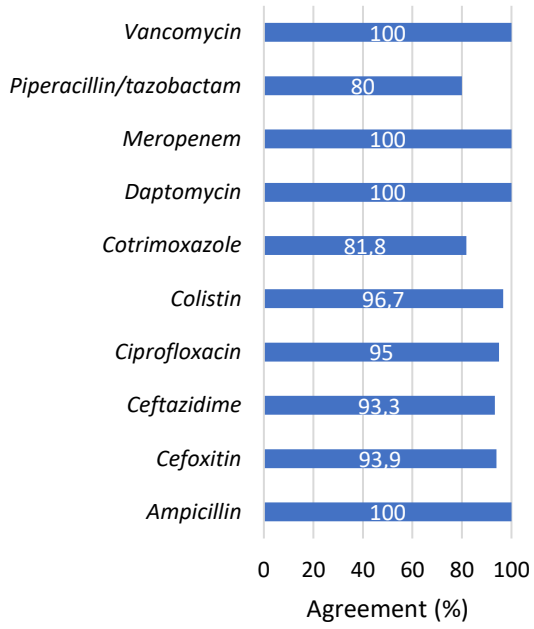
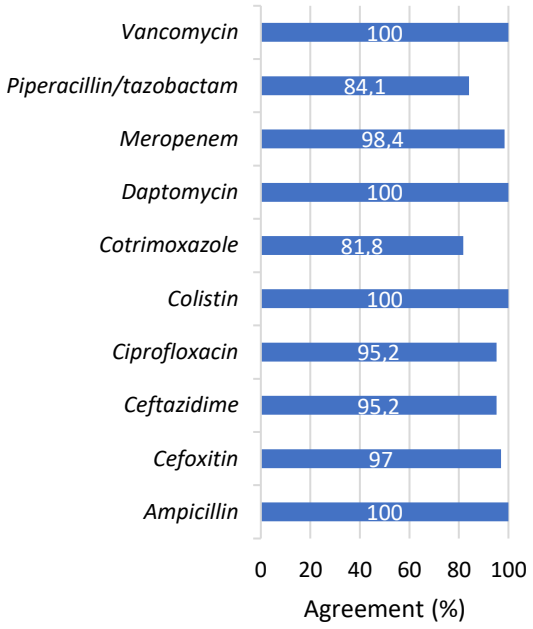


Figure 33 - RP vs. DSP AST agreement



The overall concordance between RP and DSP was higher with 94.8%. The Cohen's kappa coefficient was 0.85 (95% CI 0.79 - 0.91). One VME was registered for cefoxitin in a MR *Staphylococcus aureus* BSI. This time, RP did not exceed VME and ME limits of acceptability (VME 1.2 *vs.* ≤1.5%; ME 2.8 *vs.* ≤3%). As for PSP, among the 10 tested

molecules piperacillin/tazobactam and co-trimoxazole achieved the lowest agreement (<90%). As regards piperacillin/tazobactam there was a majority of ME and 86% occurred in ESBL-producers *Enterobacterales*. The detailed AST RP agreements and related categorical errors in comparison to DSP are summarized in Table 28 and Figure 33.

Table 28 - RP vs. DSP AST agreement and related categorical errors

Antibiotic	Test	% Agreement	VME	ME	miE	% VME	% ME	% miE
Ampicillin	6	100	0	0	0	0	0	0
Cefoxitin	33	97.0	1	0	0	4.3	0	0
Ceftazidime	63	95.2	0	1	2	0	2.4	3.2
Ciprofloxacin	63	95.2	0	0	3	0	0	4.8
Colistin	63	100	0	0	0	0	0	0
Co-trimoxazole	33	81.8	0	2	4	0	6.9	12.1
Daptomycin	33	100	0	0	0	0	0	0
Meropenem	63	98.4	0	0	1	0	0	1.6
Piperacillin/tazobactam	63	84.1	0	7	3	0	13.5	4.8
Vancomycin	39	100	0	0	0	0	0	0
Total	**459**	**94.8**	**1**	**10**	**13**	**1.2**	**2.8**	**2.8**

VME: very major error; ME: major error; miE: minor error

As regards miE, there was a tendency to overestimate the bacteria resistance for a given antibiotic in the 83.3% for RP *vs.* PSP and in the 69.2% for RP *vs.* DSP, with no underestimation of resistant phenotypes to intermediate.

Turn-around time results

MALDI-TOF MS and LST provided results in same day of sample processing. As regard the time from the starting of the test to the production of the result, among the different instrument used in the RP and SP, as expected, the RP technical time was smaller than the SP ones (Table 29 and Figure 34).

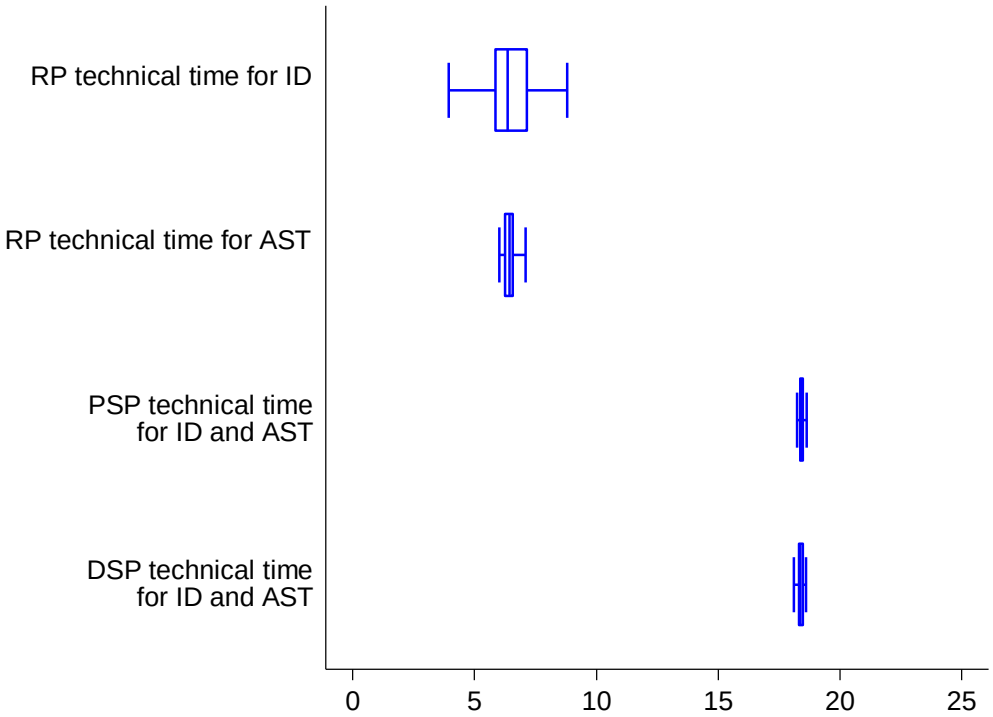
Table 29 - Protocols technical time expressed in hours

Technical time	Min	Max	p5	p25	p50	p75	p95	Mean	Std.dev.
RP for ID (N=102)	3.2	9.2	3.9	5.9	6.4	7.2	8.0	6.4	1.2
RP for AST (N=102)	6.0	8.3	6.1	6.3	6.4	6.6	7.1	6.5	0.4
PSP for ID and AST (N=99)	18.0	42.4	18.0	18.4	18.4	18.5	21.1	18.8	2.5
DSP for ID and AST (N=102)	15.3	42.6	18.0	18.3	18.4	18.5	24.1	19.6	5.2

As regard RP, the technical time to achieve ID results was slightly superior to AST and with a wider variability. Only in 7 (6.9%) cases the MALDI-TOF MS TAT exceeded the 8 hours due to the necessity of repeating the ID process.

Considering the real-life application of the protocols, the TAT was wider. TAT for ID and AST distribution according to different protocols are reported in Table 30. PSP and DSP performances were far longer in terms of response (Table 29).

Figure 34 - Boxplot of protocols technical time (hours)



As expected, RP TAT was smaller than PSP and DSP, both calculating from BC positivity and from positive BC processing. The average advantage of RP *vs.* PSP for ID was of 21.3 hours (90% CI 20.6 – 21.9) from positive BC processing.

Table 30 – Descriptive statistic of RP, PSP, and DSP TAT

TAT	Starting time	Min	Max	p5	p25	Median	p75	p95
RP for ID (N=102)	*From BC positivity*	5.7	25.6	8.0	13.1	17.2	20.0	24.6
	From positive BC processing	3.2	9.2	3.9	5.9	6.4	7.2	8.0
RP for AST (N=102)	*From BC positivity*	8.2	29.6	11.8	16.1	19.7	23.7	28.0
	From positive BC processing	8.0	12.5	8.4	9.0	9.5	10.4	11.1
PSP ID and AST (N=99)	*From BC positivity*	26.6	67.5	28.2	34.0	37.7	41.3	45.3
	From positive BC processing	25.0	54.5	25.6	26.3	27.1	27.7	29.8
DSP ID and AST (N=102)	*From BC positivity*	48.3	149.8	53.1	58.9	64.5	99.9	118.5
	From BC culture processing	48.0	145.8	49.0	50.5	51.7	98.1	101.4

RP and SP TAT from BC positivity were more heterogeneous than form BC processing (Figure 35 and Figure 36). DSP TAT was much longer than 24 additional hours to PSP TAT because the weekend could be included in the BC processing.

Figure 35 - Boxplot TAT (from BC positivity)

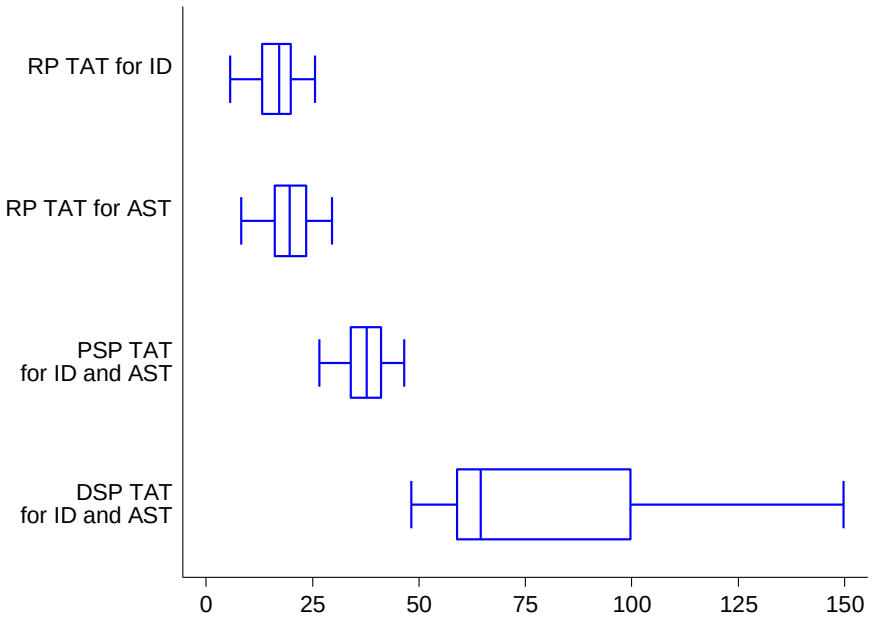
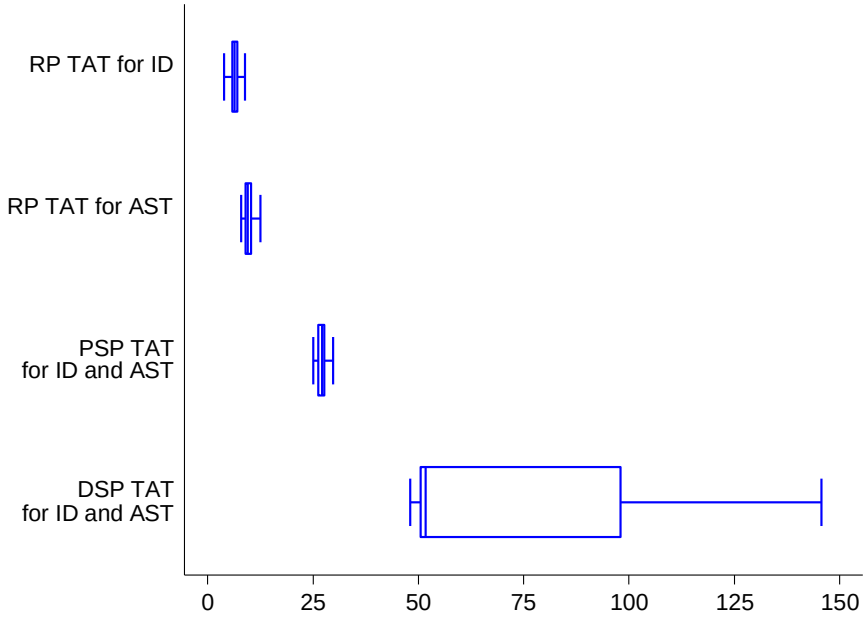


Figure 36 - Boxplot TAT (from positive BC processing)



Considering AST TAT from BC processing, the RP *vs.* PSP average advantage was of 21.1 hours (90% CI 20.5 - 21.8). As regard RP, even if the technical time for ID was longer than the AST one, however, the starting point for ID was anticipated compared to AST and began immediately after BC processing. Indeed, ID TAT never exceeded AST TAT and the ID results were available in 100% of the case before AST end for its correct interpretation.

Antimicrobial therapy survey and rapid protocol potential impact

In the absence of 3 PSP results, physicians survey could be registered for 99 (97.1%) out of 102 septic episodes. Treating physicians decisions to change the therapeutic regimen during patients hospitalization and their effective application are summarized in Table 31.

Table 31 - Potential and effective antimicrobial therapy changes during hospitalization

Antimicrobial therapy changes	Yes		No		Declared / Effective
	N.	%	N.	%	
Clinical status	5	5.1	94	94.9	*Declared*
RP	29	29.3	70	70.7	*Declared*
Short Panel PSP	29	29.3	70	70.7	*Declared*
PSP	33	33.3	66	66.7	*Declared*
PSP	31	31.3	68	68.7	*Effective*

In 12 cases the registered therapeutic change declared by physicians during the survey were not translated into practice. In the 66.7% of the cases the empiric therapy was maintained until DSP results arrival. As reported in Table 32, the declared reasons of antimicrobial regimen changes were in the majority of the cases (77.8%) related to laboratory results regardless the considered protocol and its results.

Table 32 - Declared reasons of antimicrobial regimen changes

Reason of antibiotic therapy decision	After RP results		After Short Panel PSP results		After PSP results	
	N.	%	N.	%	N.	%
Laboratory results only	41	41.4	41	41.4	43	43.4
Laboratory results and clinical status	26	26.3	26	26.3	26	26.3
Laboratory results, clinical status and other	7	7.1	8	8.1	7	7.1
Laboratory results and other	3	3.0	3	3.0	3	3.0
Clinical status and other	13	13.1	13	13.1	12	12.1
Clinical status	6	6.1	5	5.1	5	5.1
Other	3	3.0	3	3.0	3	3.0

Clinical status

As expected, the clinical status of patients changed and improved from BC sampling in the majority of patients (Table 33). The survey submitted to the treating physicians revealed that the patients clinical status, and its changes, brought to only 5.1% of modifications in the empiric antibiotic treatment. The therapeutic changes, reported in Table 33, were most frequent (80%) in patients with a stable or worsening clinical status.

The overall agreement with PSP was slight with 67.7% (90% CI 59.1 – 75.4). The potential new therapies agreed with PSP based choices in 2/5 (40%) cases. The clinical status survey agreement with other protocols is described in Table 34 and Table 35.

Table 33 - Clinical status at survey and antimicrobial therapies changes

	Clinical status at the survey		Antibiotic therapy changes based on clinical status	
	N.	**%**	**N.**	**%**
Improved	67	67.7	1	1.5
Stable	27	27.3	2	7.4
Worsened	5	5.1	2	40.0

Table 34 - Clinical status-based therapies agreement with RP and SP

Agreement	%	90% CI	
Clinical status and RP	71.7	63.3	79.1
Clinical status and Short Panel PSP	73.7	65.5	80.9
Clinical status and PSP	67.7	59.1	75.4

Table 35 - Agreement between therapeutic changes based on clinical status and other protocols

Agreement	Concordant			Discordant		
	Total	**No change**	**Changes**	**Total**	**Changes**	**Clinical status change and protocols no**
Clinical status and RP	72	70	2	27	2	1
Clinical status and Short Panel PSP	73	69	4	26	0	1
Clinical status and PSP	67	65	2	32	2	1

The agreement between RP and other protocols was >90%, but there was not statistical significance (Table 36). The antibiotic regimen changes on the basis of the protocol and their concordances are detailed in Table 37.

Table 36 - RP-based therapies agreement with SP

Agreement	%	90% CI	
RP vs. Short Panel PSP	93.9	88.4	97.3
RP vs. PSP	90.9	84.7	96.2
Short Panel PSP vs. PSP	92.9	87.1	96.6

Table 37 - Antibiotic regimen changes based on the protocol and their concordances

	Concordant			Discordant		
Agreement	**Total**	**No change**	**Changes**	**Total**	**Changes**	**RP change and SP no**
RP vs. Short Panel PSP	93	69	24	6	4	1
RP vs. PSP	90	66	24	9	5	4
Short Panel PSP vs. PSP	90	66	26	7	3	4

Rapid protocol

Between RP and PSP there was a substantial agreement with 90.9% (90% CI 84.7 – 96.2) of matching therapeutic regimens, over the expected 90%, but without statistical significance. The RP information produced 29 (29.3%) early potential targeted antimicrobial therapies, 82.8% in agreement with PSP (Table 36). In Table 37 and Figure 37 are summarized RP and PSP commonalities and differences in antibiotic regimens choices. A general representation of the agreement between the different protocol and of their CI in reported in Figure 38. Even in the subgroups analysis, reported in Table 38 and Figure 39, in the majority of the cases the agreement between RP and PSP exceeded the 90% but without statistical significance. A graphical representation is given in Figure 38 and Figure 39. The agreement between RP and Short Panel PSP did not reached the 100% due to RP AST errors.

Figure 37 - Graphic representation of RP and PSP agreement in therapeutic changes

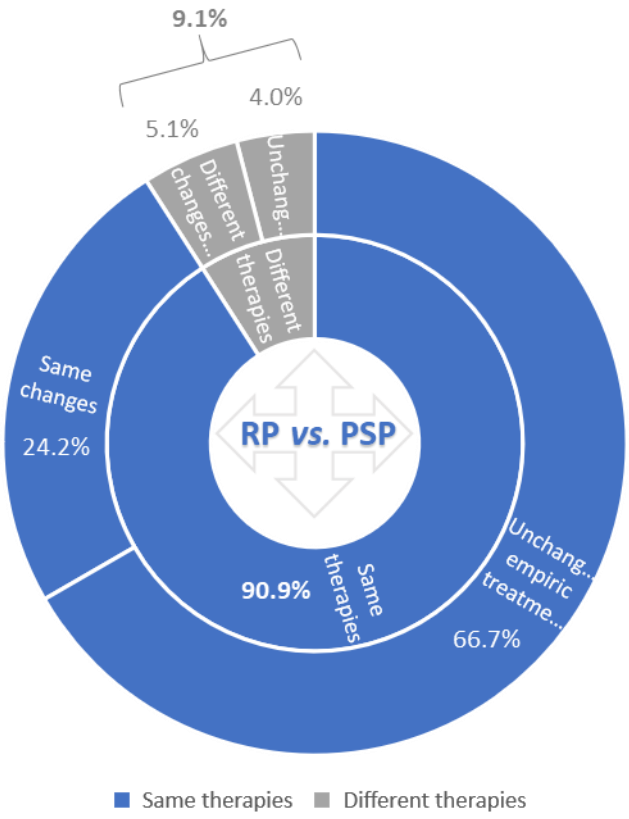


Figure 38 – Boxplot therapeutic decisions agreement and 90% CI between different protocols (PSP as reference)

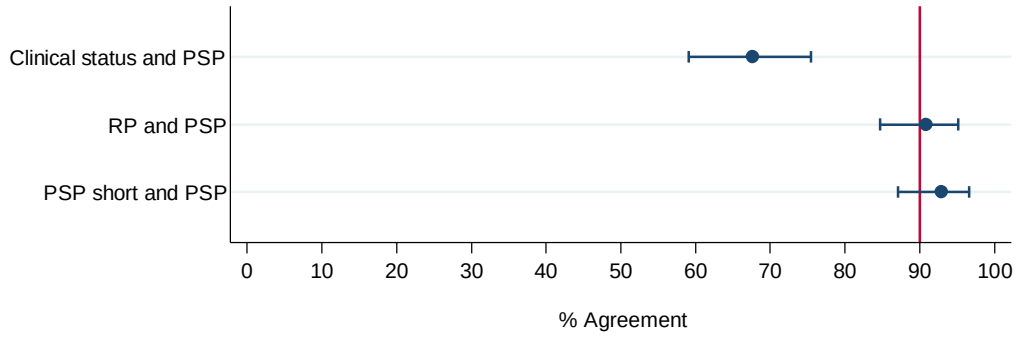
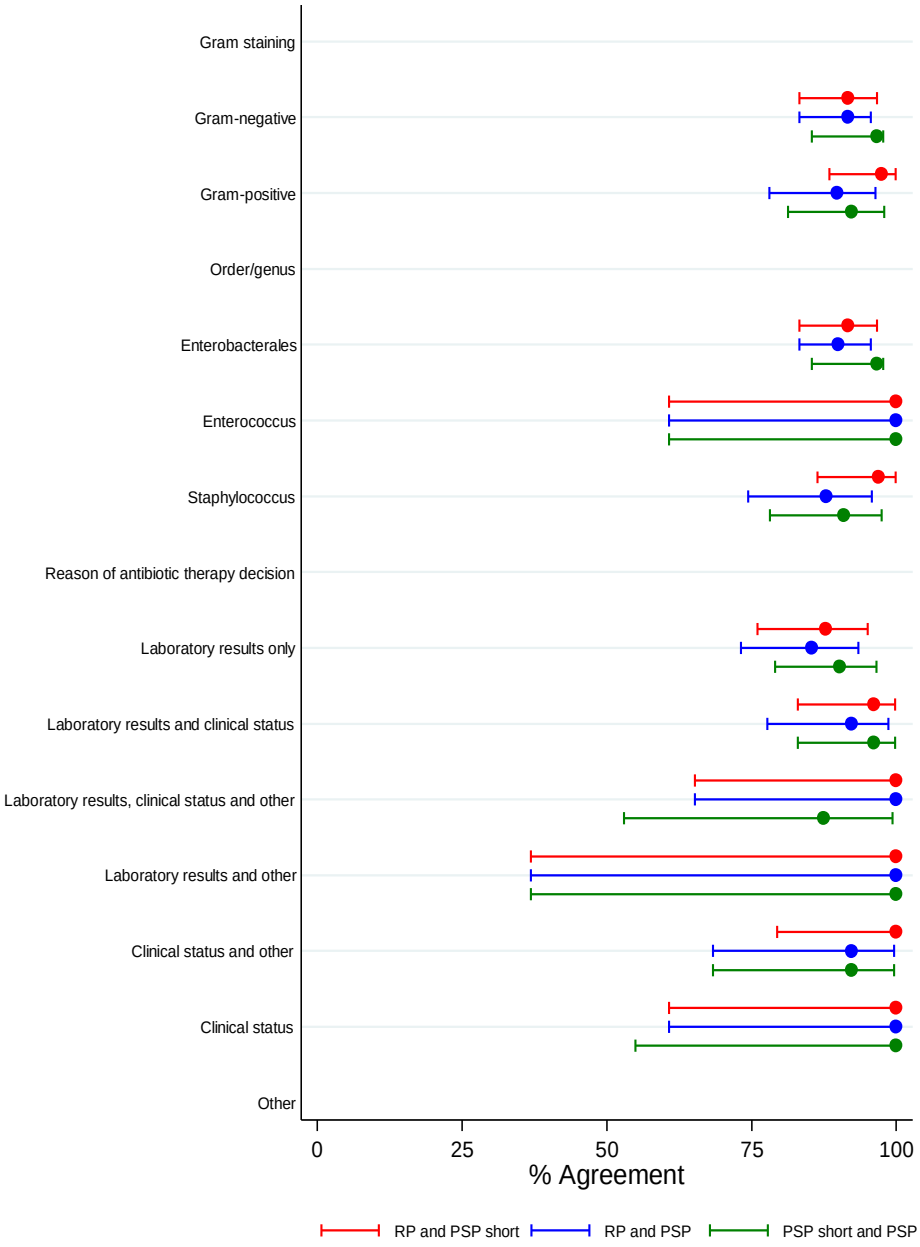


Table 38 - Subgroups analysis for RP, Short Panel PSP and PSP agreement

Variables	N.	RP vs. Short Panel PSP			RP vs. PSP			Short Panel PSP vs. PSP		
		% Agreement	90% CI		% Agreement	90% CI		Agreement	90% CI	
Sex										
Female	44	93.2	83.3	98.1	93.2	83.3	98.1	90.9	80.4	96.8
Male	55	94.5	86.5	98.5	89.1	79.6	95.1	94.5	86.5	98.5
Age										
<70	31	96.8	85.6	99.8	93.5	81.1	98.8	93.5	81.1	98.8
70 - 79	26	100	89.1	100	88.5	72.8	96.8	88.5	72.8	96.8
≥80	42	88.1	76.6	95.2	90.5	79.5	96.7	95.2	85.8	99.1
Charlson Comorbidity Index										
0	17	88.2	67.4	97.9	94.1	75.0	99.7	88.2	67.4	97.9
1	19	94.7	77.4	99.7	94.7	77.4	99.7	100	85.4	100*
≥2	63	95.2	88.2	98.7	88.9	80.1	94.7	92.1	84.0	96.8
Wards										
S.C.D.U. Medicina Interna 1	11	90.9	63.6	99.5	90.9	63.6	99.5	100	76.2	100*
S.C.D.U. Medicina Interna 2	7	85.7	47.9	99.3	85.7	47.9	99.3	85.7	47.9	99.3
S.C.D.U. Medicina Interna 3	14	85.7	61.5	97.4	85.7	61.5	97.4	92.9	70.3	99.6
S.C.D.U. Medicina Interna 4	16	100	82.9	100	93.8	73.6	97.7	93.8	73.6	99.7
S.C. Medicina Interna 5	41	95.1	85.4	99.1	92.7	82.2	98.0	92.7	82.1	98.0
Other	10	100	74.1	100.0	90.0	60.6	99.5	90.0	60.6	99.5
Gram staining										
Gram-negative	60	91.7	83.3	96.7	91.7	83.3	95.6	96.7	85.4	97.7
Gram-positive	39	97.4	88.4	99.9	89.7	78.0	96.4	92.3	81.3	97.9
Order/genus										
Enterobacterales	60	91.7	83.3	96.7	90.0	83.3	95.6	96.7	85.4	97.7
Enterococcus	6	100	60.7	100	100	60.7	100	100	60.7	100*
Staphylococcus	33	97.0	86.4	99.8	87.9	74.4	95.8	90.9	78.1	97.5
Reason of antibiotic therapy decision										
Laboratory results only	41	87.8	76.1	95.1	85.4	73.1	93.4	90.2	79.0	96.6
Laboratory results and clinical status	26	96.2	83.0	99.8	92.3	77.7	98.6	96.2	83.0	99.8
Laboratory results, clinical status and other	7	100.0	65.2	100	100	65.2	100	87.5	52.9	99.4
Laboratory results and other	3	100.0	36.8	100	100	36.8	100	100	36.8	100*
Clinical status and other	13	100.0	79.4	100	92.3	68.4	99.6	92.3	68.4	99.6
Clinical status	6	100	60.7	100	100	60.7	100	100	54.9	100*
Other	3	100	36.8	100	100	36.8	100	100	36.8	100*

*one-sided

Figure 39 - Boxplot subgroups analysis for RP, Short Panel PSP and PSP agreement



RP results could bring to a 17.2% (17/99) of early de-escalation therapies in common with PSP antibiotic changes. No carbapenem sparing could be possible, but in the 17.6% (3/17) of the cases glycopeptides administration could be stopped. The most frequent de-escalation interventions were the narrowing-spectrum and the reduction of the number of antibiotics to monotherapy. For RP and Short Panel PSP, antimicrobial therapy modifications in agreement with PSP, are described in Table 39.

Table 39 – RP and short panel PSP possible antimicrobial therapy changes in agreement with PSP

Antimicrobial therapy modifications	RP		Short Panel PSP	
	N.	%	N.	%
de-escalations	17	70.8	18	69.2
carbapenems sparing	0	0.0	0	0.0
glycopeptides sparing	3	17.6	3	16.7
oral switch	3	17.6	3	16.7
monotherapy	11	64.7	11	5.6
narrowing-spectrum	12	70.6	13	72.2
escalations	6	25.0	5	19.2
with carbapenems	4	66.7	3	60.0
with glycopeptides	1	16.7	1	20.0
intravenous switch	0	0.0	0	0.0
broadening-spectrum	6	100.0	5	100.0
combination therapy	1	16.7	1	20.0
(other) targeted therapy	1	4.2	3	11.5

The 5 discordant therapeutic changes between RP and PSP occurred in 4/5 cases of ESBL-producers *Escherichia coli* with RP ME in ceftazidime and ciprofloxacin (n. 1) and in piperacillin/tazobactam (n. 3). In 2/4 cases the discrepancy regarded a not reported drug in the Gram-negative panel belonging to the same class (i.e., ertapenem instead of meropenem). In 1/5 case the discrepancy regarded a CoNS, probably a contaminant, and in particular ciprofloxacin, a drug not tested in Gram-positive RP panel. Moreover, in all the considered cases the RP discrepancies brought to the escalation of the antimicrobial therapy.

Short panel preliminary standard protocol

The agreement of Short Panel PSP with PSP was of 92.9% (90% CI 87.1 - 96.6), higher than RP, but, even in this case without statistical significance. Thanks to short panel PSP results, 29 (29.3%) early antibiotic regimens modifications could be done, 89.7% in accordance with PSP. The difference in performance of RP and Short Panel PSP was due to 2 cases of ESBL-producers *Escherichia coli* in which RP recorded ME in the molecules

chosen for therapy, ciprofloxacin and piperacillin/tazobactam. De-escalation was possible in 18/26 (69.2%) concordant changes with PSP. Even in this case, the most frequent de-escalation interventions were the narrowing of the antibiotic therapy spectrum and the reduction of the number of antibiotics to monotherapy.

Mortality rate

The registered in-hospital mortality rate was of 6%. All the patients deceased for the underlying diseases of which the 66.7% were malignancies. The totality was over 60 years-old (average age 74 years) with 83.3% of males. In 83.3% (5/6) of the cases the RP-based, short panel PSP-based and PSP-based therapeutic decisions agreed, and 3 antimicrobial regimens could have correctly changed in advance on the basis of RP results. In one case PSP therapeutic decision was different and involved a not tested drug. The 30-day mortality rate was of 10% with 60% of males and an average age of 75 years. Only 5% of these patients died during hospitalization. The causes of death in the other cases were not registered. Similarly to in-hospital mortality rate, in these cases the therapeutic decisions had a 80% of agreement with the 50% of possibility to anticipate the antibiotic changes on the basis of RP results.

Discussion

The issue of rapid diagnostic tests is one of the most important challenge in Clinical Microbiology in the last 10 years. In the 2013 the IDSA, in a policy paper called "*Better Tests, Better Care: Improved Diagnostics for Infectious Diseases*", urged to *"translate new technologies into practical tests"*[72]. The main obstacles that new technologies, new tests, have to overtake are the TAT reduction, the necessity of invasive specimens, the application to particular severe clinical conditions or syndrome (*e.g.* central nervous system infections) and to selected patients (*e.g.*, paediatric or septic patients), reaching a high level of negative predictive value to rule out infection or MDRO[72,73]. BSI are one of the most advocated field of intervention. Their important impact on patients morbidity and mortality rates, on antibiotic therapies administration, on the antimicrobial resistance phenomenon spreading, on hospital and moreover Public Health costs, makes BSI an important target for Clinical Microbiology and AS programmes. The availability of rapid tests alone, however, does not necessary impact on patients clinical outcome, since their use without rapid communication of results or in absence of AS programmes, for example, reduces or cancels their positive impact on patients management[56,57]. To enhance the strengths of these methods and, at the same time, improve patient outcomes and reduce BSI burden, rapid tests need to be integrated into privileged diagnostic workflows[59]. These rapid workflows, also called "fast-tracks", should be designed in strict collaboration with AS groups[63].

Numerous information is needed to better plan and set-up these fast tracks into the real clinical context. First, there is the need to restrict the access to selected patients. Most of the tests used to speed-up the microbiological diagnostic process are single-sample cartridge tests, like POCT, or have a limited number of samples that can be loaded at the same time or in the same working day[46,74]. This reason, in addition to the necessity to contain additional Public Health costs, imposes that a selection process based on disease severity, urgency of intervention or time-dependent illness, should be adopted[59,75]. In this study, we decided to investigate RP potentialities in septic patients admitted to Internal Medicine, Geriatric wards and Emergency Unit. Despite 2016 released Sepsis-3 definition, in this clinical context, SIRS criteria, considering clinical and biochemical

variables, were preferred to qSOFA score in sepsis definition. The choice was guided by the adoption of Sepsis-2 definition in the hospital empiric antimicrobial therapy manual, published close to Sepsis-3 release, guiding the empiric antibiotic therapy in Medical wards, and for the enhanced sensitivity for organ dysfunction in comparison with qSOFA score, especially in non-ICU settings[9,69,76–79] A higher sensitivity allows an early recognition of patients with a time-dependent syndrome like sepsis avoiding their transfer to an Intensive Care Unit thanks to a prompt therapeutic intervention. Moreover, in our case studies, in the 56.7% of the patients qSOFA score could not be calculated because at least one fundamental parameter was missing: the respiratory rate, was rarely registered on medical record, frustrating qSOFA practical application to bedside. Even though patients selection process for fast-track Microbiology needs validated multi-parametric scores, taking into account clinical, microbiological and biochemical variables, SIRS criteria could be considered a sensitive method for septic patients interception in non-ICU setting without reaching the saturation of the rapid diagnostic tests[59].

Another important point in the set-up of the diagnostic protocol is the knowledge of the local epidemiology. Pathogens to be covered by the rapid tests, the antimicrobials to be tested in the panels, are direct consequences of the infectious disease to be treated and of the local microbiological ecology[28].

The application setting of the RP was a Medical ward setting in which the main BSI aetiologic agents were represented by CoNS, *Escherichia coli* and *Staphylococcus aureus* in concordance with literature data[80–83]. A low prevalence of multidrug-resistant *species*, frequently associated to carbapenem-resistance, such as *Klebsiella pneumoniae* (range 3.9% - 5.2%), *Pseudomonas aeruginosa* (range 1.1% - 3.2%) and *Acinetobacter baumannii* (range 0.6% - 0.9%) was registered. Data regarding antimicrobial-resistance pattern of the 3 most frequent isolated *species* showed a low endemicity of carbapenems and glycopeptides resistances. Third-generation cephalosporins and carbapenems resistances of *Escherichia coli* were in line with literature data[82,84,85]. The proportion of MR *Staphylococcus aureus* was higher in comparison with Italian EARS-Net data in the period 2016 – 2018, but with a descending trend, and a value returned

in range, in 2019. MR *Staphylococcus epidermidis*, on the contrary was abnormally high even if comparable data on Medical wards BSI are scarce. RP's BSI pathogens coverage through the considered years was excellent reaching a 99% of targeted non-fastidious bacteria in 2019. The extended coverage of the RP is one of its major advantage in comparison to other protocols, in particular those based on molecular methods[29,31,74,86]. MALDI-TOF MS library could ensure a correct ID for more than 2000 different *species*, a firepower unparalleled by any other ID methods routinely applied in CML activity. On the other side ALFRED60AST system used for AST allows to customize the antimicrobial panels to be tested on the basis of user's needs, such as the isolated pathogen, when known, or more in general, such as local epidemiology. Despite over 50% of the enrolled patients received an antimicrobial therapy in the previous 30 days, 47.1% had a previous hospital admission in the previous 6 months, and the elevated number of comorbidities (63.3% Charlson Comorbidity Index ≥2), the registered endemicity of MDRO was low especially for Gram-negative bacteria. In this setting the choice of a phenotypic method for AST determination, with multiple tested drugs, produces a wider range of therapeutic options expendable in clinical practice in comparison to immunochromatographic or molecular tests[35,87].

Data on the selected wards and population in the period 2016 – 2019 were stable and comparable to the enrolled subpopulation. As regards glycopeptide and carbapenems administration trend and DRG average standardized charge, the results highlight the presence of 2 wards with major intensity of cure, *S.C.D.U. Medicina Urgenza*, of course, and *S.C.D.U. Medicina Interna 2*, an Internal Medicine ward with an emergency vocation compared to the other Internal Medicine wards. However, the subgroups analysis didn't underline significant differences in patients therapeutic management both for RP and SP on the basis the selected wards.

The application of ALFRED60AST system in the fast-track Microbiology presents a series of plus. It's a semiautomatic instrument with limited manual operations from sample loading to the production of results, and therefore the operator workload is reduced. The number of samples that could be loaded at the same time is related to the number of tested antibiotics: with a panel of 6 molecules, up to 7 samples could be loaded at the

same time, more than its principal competitors[48,74]. Another advantage of ALFRED60AST system is the standard inoculum for AST determination. Many other rapid phenotypic tests based on direct AST from positive BC could be affected by the inoculum effects[32,41,44]. Moreover, the prepared standard inoculum could be ready to use for other diagnostic purposes (*e.g.* additional conventional phenotypic tests for molecules not included in commercial AST panels), in less than 1 hour and 30 minutes. Moreover, as regards the appropriate standardized bacterial inoculum, in our cases probably an inadequate one was the cause of 2/3 failed PSP results.

Another plus of the RP is that there is no need of additional samples drawing from septic patients. The entire protocol works on BC bottles withdrawn during patients septic episodes as part of the normal, and mandatory, BSI diagnostic process[6,14,15]. The analysis of the number of positive BC sets is pivotal for the critical revision of potentially contaminated samples. This process, however, is not possible for example with molecular methods directly applied to peripheral blood.

The weaknesses of ALFRED60AST system, however, are noteworthy. The system lacks an effective expert system able to interpret the AST results. Considering the staff workload, the antibiotics are lyophilic and must be carefully solubilize before the starting of the tests even if, once solubilized, they could be used up to 5 times in a period of 5 days if stored at 4°C in the instrument refrigerated zone (except for meropenem, 3 days).

As regard RP accuracy, MALDI-TOF MS, as expected, it is confirmed a reliable method for pathogens ID with 97% of correct identification at *species* level in agreement with conventional methods and no registered mismatch[53,54,88]. The data were available for the correct interpretation of AST in the 100% of the cases. The short incubation on solid medium of positive BC in our CML reality, provides results in restrained time, about 6.4 hours, with a limited workload for laboratory personnel particularly in presence of an elevated number of positive BC to be processed.

As regards AST performed with ALFRED60AST, the agreement with PSP reference conventional methods was over 93%, comparable or even better than those reported in literature (90.5% *Barnini et al.* in 2016, 90.3% *Giordano et al.* in 2018, 88.1-92.2% *Boland*

et al. in 2019, 94.1% *Mantzana et al.* in 2021)[48,49,89,90]. The RP agreement with DSP, performed with standardized reference methods on isolated colonies from over-night incubation, was greater than PSP (94.8% *vs.* 93.5%) and the percentages of errors were within the range of acceptance[71]. Only one VME was registered as regards cefoxitin in RP-DSP comparison. Unfortunately, the error occurred in a BSI caused by MR *Staphylococcus aureus* but without influencing the empiric treatment already based on vancomycin. Piperacillin/tazobactam and co-trimoxazole had the lowest percentages of agreement in comparison to DSP with 84.1% and 81.8% respectively. For piperacillin/tazobactam a low agreement with reference methods was already observed in other studies (*e.g.* 77.1% *Giordano et al.* 2018)[48]. In our cases the majority of errors were linked to overestimation of antimicrobial resistance (ME) in ESBL-producers *Enterobacterales*. Therefore, the therapeutic consequences were limited: in one case ertapenem was administrated instead of meropenem and in the other one piperacillin/tazobactam therapy continued despite RP results. Given the scarce performance of ALFRED60AST system in piperacillin/tazobactam resistance determination, the recent EUCAST introduction of the area of technical uncertainty for the binomial *Enterobacterales*-piperacillin/tazobactam, and the limited role of this drug in BSI of ESBL-producers *Enterobacterales*, it could be considered to avoid its determination in favour of other antibiotics such as the more used aminoglycosides[91,92]. Moreover, on the clinical side, the frequent adoption of a combined regimen based on piperacillin/tazobactam and an aminoglycoside (*e.g.* gentamycin) could reduce the potential dramatic effect on patients management of other errors (*e.g.* VME) while waiting for definitive AST results. As regards co-trimoxazole, it was used in only 5.9% of therapeutic regimens during enrolled patients hospitalization despite its conserved activity against Gram-positive bacteria. Co-trimoxazole errors were in the 66.7% miE with 75% overestimation of resistance (from S to I) and no effect on therapeutic choices. Even in this case the possibility of replacing co-trimoxazole in Gram-positive panel could be considered in favour of other drugs (*e.g.* linezolid). Generally, the AST error tendency was to overestimate antimicrobial resistance instead of underestimate (2.8% ME *vs.* 1.2% VME and 69.2% miE from S to I and from I to R). This could be considered an

additional reason to consider RP safe in its routinely application regardless its suboptimal performance for a limited number of antibiotics.

As regards TAT evaluation, incubation time on solid media for achieving MALDI-TOF MS ID could not be shortened more than 3-5 hours, ALFRED60AST system time to reach 0.4-0.6 *McFarland* could vary but the time to interpret bacterial growth curves is fixed to 3-5 hours on the basis of the tested drug. The median technical time for both tests was <8 hours configuring them as rapid tests, as expected, with TAT in line with other studies[21,48,49,53,54,88,89]. As regards MALDI-TOF MS and ALFRED60AST system real application, instead, the TAT was necessary longer, and this time should be considered for real-life protocol impact evaluation. The TAT from BC positivity is the most influenced by laboratory organization and therefore less comparable with other settings. On the contrary, TAT from positive BC processing is comparable and at the same time takes into account the real-life application of the methods. The RP gain in terms of hours, in comparison with PSP, was considerable with more than 21 hours. The real-life TAT, however, could be further reduced if patients selection process would be integrated in the BC request and not performed by CML staff after BC positivity as executed in the study for patients enrolment.

Moreover, a TAT reduction in AST determination has a potential role, not only from a therapeutic point of view, but also on infection control policies. Considering ALFRED60AST tested ceftazidime, cefoxitin, vancomycin and meropenem as warning lights for the potential presence of MDRO, RP could have correctly detected the presence of the 100% ESBL-producers *Enterobacterales,* the 75% of MR *Staphylococcus aureus*, the 100% of VRE and the 100% of CPE. RP could allow the adoption of contact precaution in advance respect to conventional diagnostic test with a potential limitation of MDRO spreading in 24.2% of the cases.

As regards antimicrobial decisions survey, considering the study design, RP reports were not freely available to physician and the survey timing choice was made to avoid any influence on patient management by RP data. The choice of recording the potential RP-based antimicrobial regimens instead of putting directly into practice the protocol, was

due to multiple reasons. Firstly, the RP agreement with reference methods in use in our laboratory was not tested before. Moreover, it was not experienced by our physicians. As reported for molecular methods results, a possible suspicion on the reliability of the results could underestimate the impact of the new fast track[93]. In support of this hypothesis, we registered 12 cases in which the declared therapies were not administered in while waiting of the definitive microbiological report.

Patients clinical status alone could be responsible of only 5% of antibiotic changes. Even though clinical status is part of the therapeutic decision process, AST results were fundamental to reach a targeted therapy. RP data could help the treating physicians to change the antimicrobial therapy in advance of more than 21 hours compared to PSP. In the majority of the cases the empiric treatment was maintained, but more than 1 patient in 5 could benefit of a prompt targeted therapy in agreement with conventional reference methods. An Infectious Disease specialist review of the cases, especially those with no changes of empiric therapy regardless AST results, will be object of a future study with the aim of revealing the potential enhanced impact of RP on patients management[94]. Of notice, the most frequent treatment modification was de-escalation. Glycopeptide sparing could be reached in 3 cases with potential positive effects on the selection of Gram-positive MDRO, the most represented MDRO population in these selected wards. If the RP had been free from errors, that is, equal to short panel PSP results, the benefit of an early targeted therapy could reach more than 1 in 4 patients. Other studies have evaluated the accuracy and TAT of the combination of MALDI-TOF MS and ALFRED60AST, but, at our knowledge no previous study have investigated their potential impact in clinical management of patients in a real-life setting[48,49,55,89,90,95]. Data on rapid combined methods impact, indeed, are few and difficult to compare. *Verroken et al.* in a masterpiece study published in 2016, evaluated the clinical impact of different combination of MALDI-TOF MS and phenotypic rapid tests applied to BSI but the CML organization, the setting of application and the local epidemiology were different or not specified[87]. The SPEED study analysed the potential clinical impact of the combined method *Accelerate Pheno system* (*Accelerate Diagnostics, USA*) only in Gram-negative therapeutic management, but the study was retrospective[96]. Various

types of studies, epidemiological setting, number and tested antibiotics, reference methods, EUCAST breakpoint and categories changing during the years, settings of application and recorded impact/outcome measures are the main bias to face.

For the previous reported reasons, also our study has some limitations. An important study limit is its observational nature. A randomized controlled trail (RCT) should be designed to better understand the real impact of this RP. In comparison to molecular methods, its application is linked to only monomicrobial positive BC infected by non-fastidious bacteria. However, as regards combined methods, currently, only *Accelerate Pheno system* could produce results in polymicrobial BC and solely about ID without executing AST[47,48,97]. Another limit of this study regards the batching process of samples that constitutes a compromise between the large number of positive BC, the limited staff resources and the opening working hours of the laboratory. The working hours, indeed, are another important bias in RP evaluation. Time and days limits, in the selection criteria, were chosen due to laboratory workload and staff and working hours restrictions particularly during holidays. At the CML *S.C. Microbiologia e Virologia U.*, about 25000 blood cultures sets are processed annually, with an average of 30 positive blood culture bottles per day. From Monday to Friday, during laboratory opening days, blood cultures are processed in batch mode with 2 sessions, one in the morning by 10:00 and one in the afternoon by 15:00. On holidays, due to personnel limitations, only microbiological emergencies were performed, and blood cultures are analysed in batch once a day. After 10:00 on working days and on holidays, ID and AST of the microorganisms from positive blood culture bottles are performed from only isolated colonies after overnight subcultures incubation. The study sample selection criteria allowed a fair comparison between RP and PSP balancing the pros and cons of both diagnostic protocols and allowing to evaluate them at their maximum effectiveness according to laboratory workflow. Considering the obtained results, a median RP TAT <10 hours from blood culture processing, the maximum advantage of RP application, however, could be reached on the penultimate working days. The impact of the RP is linked not only to CML operating hours, but also to the availability of physicians ready to process RP information in the late afternoon and translate it into prompt targeted

therapies[87]. A 24/7 organization on both sides, the CML and wards, could be the best solution to extend the potential benefits of every BSI microbiological fast-track[32,87,93]. Unfortunately, our laboratory at the moment doesn't belong to the 13% of the European microbiological laboratories that process positive BC 24/7 and not even to the less than 5% that produce 24/7 microbiological reports[19]. On the other side, the physicians in the wards should be sensitized to review antibiotic therapies as soon as laboratory results become available.

Conclusions

The most important aim of AS programmes is to optimize the use of antibiotics. The CML is directly involved in the promotion of correct antimicrobials administration through the aetiological diagnosis of infections and the determination of pathogens AST. The CML also impacts indirectly on AS programmes through the production of epidemiological data that are necessary to guide the empiric antibiotic therapies. Sepsis and BSI are one of the main fields of application of AS programmes due to the impact of early targeted antimicrobial therapies on hospitalized patients morbidity and mortality rates and on Public Health direct and indirect costs. Fast-track Microbiology, through the early pathogens ID and AST, provides essential information for switching from empiric to a targeted antibiotic therapy meeting both outcome and AS goals.

To our knowledge this is the first study that investigated the accuracy, TAT and potential impact on patients therapeutic management in a real-life non-ICU setting of a rapid diagnostic protocol for BSI based on two combined techniques, MALDI-TOF MS for microorganisms ID and LST for AST determination. The RP demonstrated an excellent agreement with reference methods in use in our laboratory to support its safe application in clinical practice. The potential "*therapeutic TAT*" reduction was remarkable with >21 hours of possible gain, and additional time saving could be reached improving patients selection process, CML opening hours and the sensitivity of clinicians to revise antimicrobial therapies as soon as laboratory results become available[81]. However, the RP impact on clinical management of patients revealed that more than 1 in 5 patients could already benefit of an early targeted antimicrobial therapy and in more than 1 in 6 of de-escalation with sparing of important broad-spectrum antimicrobials. Moreover, the reduced TAT for AST profile determination could produce an added value allowing to put in place infection control policies in advance in more than 1 in 5 patients with a possible positive effect on antimicrobial resistance phenomenon spreading. However, to further investigate RP real impact on antimicrobial therapies administration and patients outcome, a RCT should be conducted.

Appendix

ADDITIONAL TARGET POPULATION, WARDS SETTING AND PRESCRIPTION ANALYSIS DATA

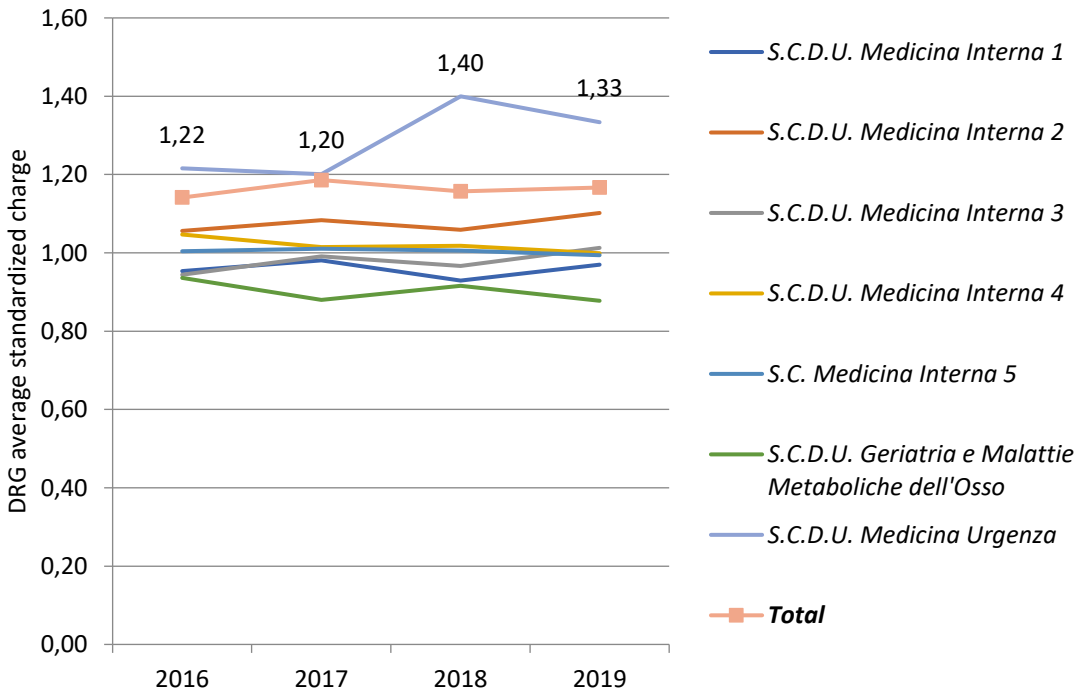
In Table 40 are reported age population data for the period 2016 – 2019. No significant differences were registered during the considered time lapse.

Table 40 - Population age in the selected ward over 2016 - 2019

Year	Min	Max	p50	p25	p75
2016	14	106	78	69	84
2017	14	107	78	69	85
2018	15	103	78	69	84
2019	14	104	78	69	85
Total	***14***	***107***	***78***	***69***	***84***

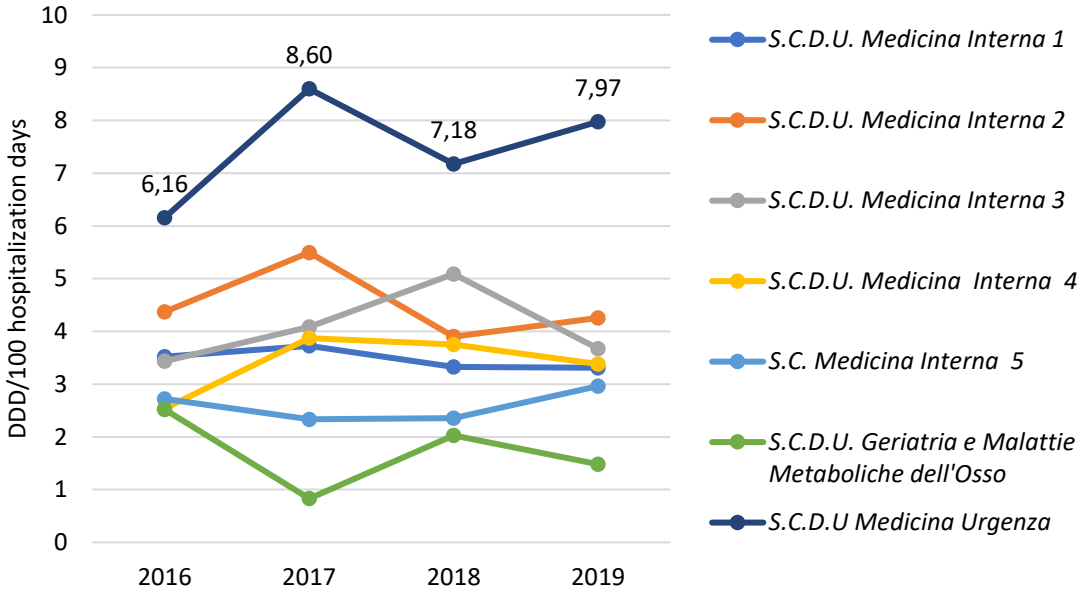
As reported in the text above, DRG average standardized charge was stable from 2016 to 2019 as regards the considered wards together. However, as expected, *S.C.D.U. Medicina Urgenza* had constant higher DRG average standardized charge values.

Figure 40 - DRG average standardized charge over the period 2016 - 2019



In Figure 41 are reported vancomycin administration data divided by wards. Several peaks and decreases were registered in each ward, but the trend was stable. *S.C.D.U. Medicina Urgenza* registered a major vancomycin prescription rate in comparison to the other wards.

Figure 41 - Vancomycin administration in the selected wards over 2016 - 2019



STANDARD PROTOCOL ACCURACY

The performances of PSP were also evaluated in comparison with DSP. The agreement between PSP and DSP was of 96.4%, higher than RP and DSP (Cohen's kappa coefficient: 0.89; 95% CI 0.84 - 0.94). The number of ME was significantly lower than reported for RP (0.6% *vs.* 2.8%), however, the VME rate was higher (5.2% *vs.* 1.2%). The majority of the VME (3/4) regarded piperacillin/tazobactam and only 1 case cefoxitin in a BSI caused by *Staphylococcus epidermidis*. None of the tested antibiotics had an agreement lower than 90%. As regards miE, in contrast to RP, there was the tendency to underestimate the bacteria resistance in most of the cases (60%). As regards the errors acceptability range, PSP exceeded the threshold only for VME (5.2 *vs.* ≤1.5%). Data of PSP and DSP agreement and related categorical errors are reported in Table 41.

Table 41 - PSP vs. DSP AST agreement and related categorical errors

Antibiotic	Test	% Agreement	VME	ME	miE	% VME	% ME	% miE
Ampicillin	6	100	0	0	0	0	0	0
Cefoxitin	33	97.0	1	0	0	4.5	0	0
Ceftazidime	60	96.7	0	0	2	0	0	3.3
Ciprofloxacin	60	95	0	0	3	0	0	5
Colistin	60	96.7	0	2	0	0	3.8	0
Co-trimoxazole	33	97	0	0	1	0	0	3
Daptomycin	33	100	0	0	0	0	0	0
Meropenem	60	98.3	0	0	1	0	0	1.7
Piperacillin/tazobactam	60	90	3	0	3	75	0	5
Vancomycin	39	100	0	0	0	0	0	0
Total	**444**	**96.4**	**4**	**2**	**10**	**5.2**	**0.6**	**2.3**

*VME: very major error; ME: major error; miE: minor error

In Figure 42 and Figure 43 are graphically summarized the AST overall agreements and categorical errors between the different protocols.

Figure 42 - Protocols AST agreement

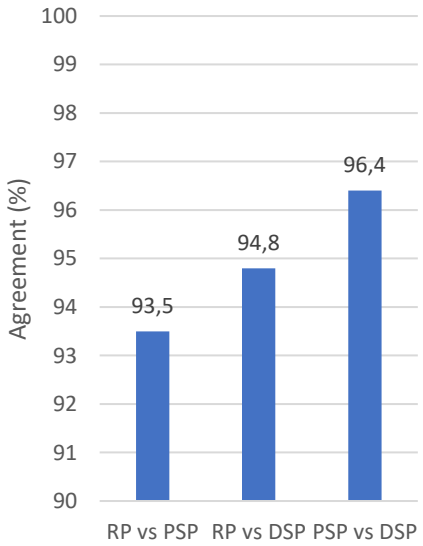
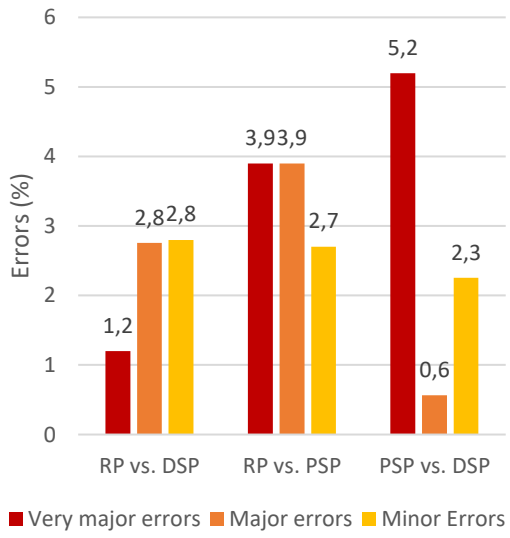
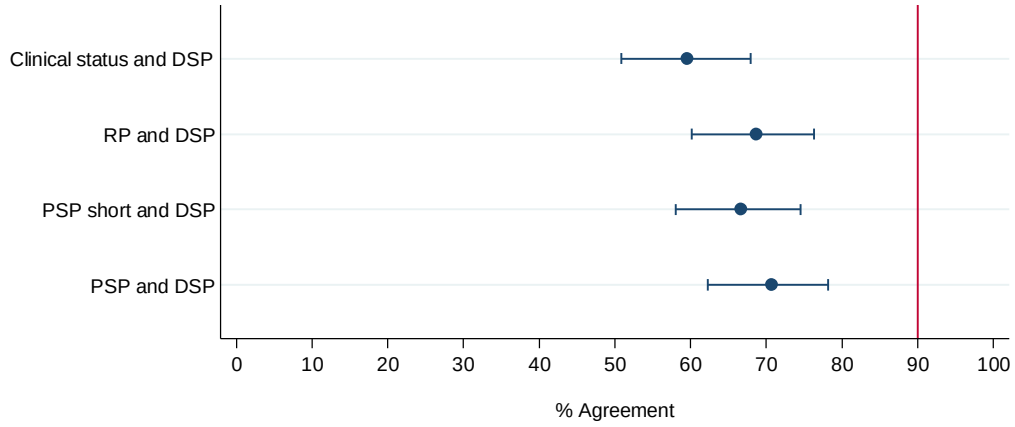


Figure 43 - RP and SP percentages of AST categorical errors



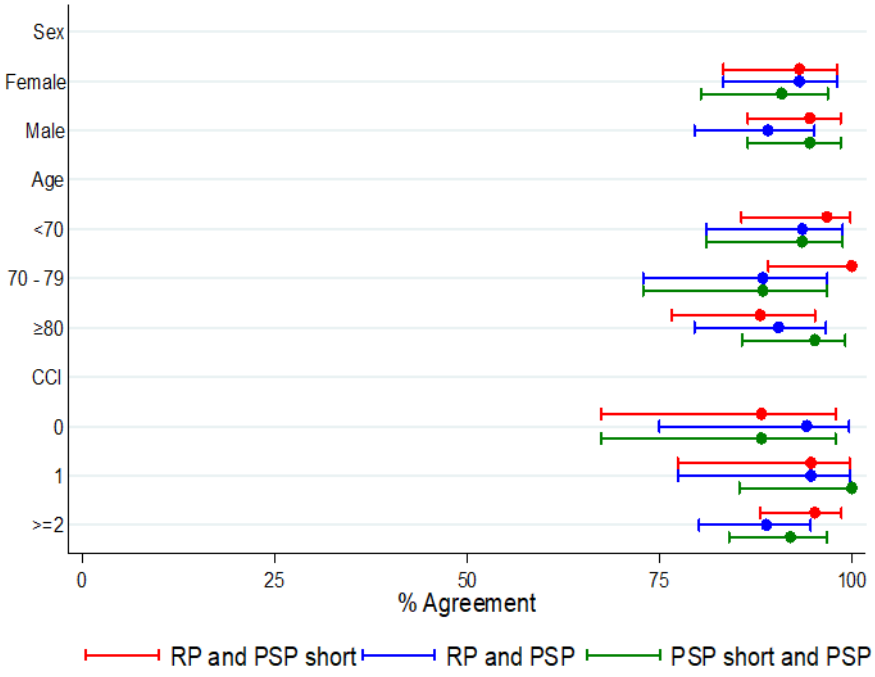
A comparison of the therapeutic decisions agreements between clinical status, RP, short panel PSP, PSP and DSP used as reference was performed and the boxplot representation with 90% CI is reported in Figure 44. However, even if RP and PSP agreement were similar (RP *vs.* DSP 68.7%, 90% CI 60.2 – 764; PSP *vs.* DSP 70.7%, 90% CI 62.3 – 78.2), the time between PSP and DSP results arrival was considerable in particular when holidays were considered. Clinical status evolutions, results of instrumental tests or biomarkers trends could be important bias in the evaluation of the therapeutic impact of RP if compared with DSP-based decisions, therefore, these data even if analysed, were not discussed in the text above.

Figure 44 - Boxplot therapeutic decisions agreement and 90% CI between different protocols (DSP as reference)



In Figure 45 is reported a boxplot subgroup analysis for RP, short panel PSP and PSP agreement, considering population features such as sex, age, and the Charlson Comorbidity Index.

Figure 45 - Boxplot subgroups analysis for RP, short panel PSP and PSP agreement



CCI: Charlson Comorbidity Index

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