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Recent advances in biochemical and molecular diagnostics for the rapid detection of antibiotic-resistant *Enterobacteriaceae*: a focus on β -lactam resistance

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ABSTRACT

Introduction: Drug resistance among bacteria is a scourge to patients and infectious disease and infection control specialists. The rapid detection of resistance is a challenge for clinical microbiologists who wish to prevent deleterious individual and collective consequences such as (i) delaying efficient antibiotic therapy, which worsens the survival rate of the most severely ill patients, or (ii) delaying the isolation of the carriers of multidrug-resistant bacteria and promoting outbreaks; this last consequence is of special concern, and there are an increasing number of approaches and market-based solutions in response.

Areas covered: β -lactams are the cornerstone of numerous empirical and definitive antimicrobial strategies. From simple, cheap biochemical tests to whole-genome sequencing, clinical microbiologists must select the most adequate phenotypic and genotypic tools to promptly detect and confirm β -lactam resistance from cultivated bacteria or from clinical specimens. Here, the authors review the published literature from the last 5 years about the primary technical approaches and commercial laboratory reagents for these purposes, including molecular, biochemical and immune assays. Furthermore, the authors discuss their intrinsic and relative performance, and we challenge their putative clinical impact.

Expert commentary: Until the availability of fully automated wet and dry whole genome sequencing solutions, microbiologists should focus on inexpensive biochemical tests for cultured isolates or mono-microbial clinical specimen and on using the expensive molecular PCR-based strategies for the targeted screening of complex biological environments (such as stool or respiratory tract clinical specimens).

KEYWORDS

Antimicrobial resistance; gene detection; β -lactamase; ESBL; MALDI-TOF; whole genome sequencing; real-time PCR; microarray assay; electrospray ionization mass spectrometry

1. Introduction

1.1. General statements

Several factors are key elements with respect to the emergence of new bacterial agents, such as the acquisition of virulence factors, an increasing capacity for rapid spreading, and the development of antimicrobial resistance [1]. This last property has broken the 'antibiotic dream' that occurred after the discovery of penicillin, and it defies the 20th century belief that natural, semi-synthetic, and synthetic drugs might definitively treat any type of bacterial infection [2]. In the USA, the number of patients who are infected with antibiotic-resistant bacteria has reached 2 million people each year, 23,000 of whom had fatal outcomes [3]. β -Lactams remain the cornerstone of antibacterial treatments [4]. We are now facing a continuous race between the marketing of new β -lactam compounds and the *in vivo* selection of β -lactamases with increased or wider hydrolytic activities, particularly in gram-negative bacteria [1,4]. *Enterobacteriaceae* and, more specifically, *Escherichia coli*, include the most important sources of

severe bacterial infections in humans for illnesses such as bloodstream infections [5]. From a human perspective, our ability to detect these resistant bacteria at the bedside of infected patients and to optimize antibiotic stewardship **are among** the essential steps in controlling the vicious circle of antimicrobial resistance. Here, we focus on advances in the rapid detection of β -lactam resistance amongst the *Enterobacteriaceae*.

1.2. What is rapid detection testing?

A rapid detection test should provide results within a two-hour turnaround time (TAT) according to the workflow observed in many clinical laboratories [6]. We can distinguish between the standard analytical time and the current TAT. Beyond the bench procedure in the laboratory, the TAT includes the time between the prescription of the test and the availability of the results to clinicians. Point-of-care tests (POCTs) that deliver results within 30 **min** of specimen collection could be performed at the patient's bedside by a

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physician or nurse. As a reminder, the culture-based techniques used as a reference here have a TAT of 18 h or longer. Moreover, the complete process of a culture-based approach requires selective screening media, antimicrobial susceptibility testing, and confirmatory tests; each of these steps lasts 16–20 h, which corresponds to the need to obtain a bacterial culture. Consequently, all the methods that appear in the literature as ‘detection tests’ but require overnight growth to produce results are outside the scope of this work. This is the case, for example, for the Carbapenemase Detection Set® used to detect carbapenemase-producing bacteria (MAST-CDS, Mast Group, Merseyside, U.K.) [7]. Beyond carbapenemase detection, the use of specific inhibitors allows for the identification of the different enzyme subgroups on the same day or on the following day at a low additional cost [8]. As further reported, the ongoing development of automated digital analysis could accelerate the detection of bacterial growth and may decrease the TAT of these methods [9].

1.3. Relevance of the subject

1.3.1. Contribution of rapid detection tests to antimicrobial stewardship

Next to infection control measures, antimicrobial stewardship is the cornerstone of the fight against multidrug-resistant microorganisms (MDRO). The implementation of an antimicrobial stewardship program is based on a series of measures such as the elaboration of empirical antibiotic therapy procedures, skilled expertise among infectious disease consultants, control-of-last-resort antimicrobial compounds, and the availability of laboratory-based tools to confirm the bacterial origin of sepsis (e.g. the procalcitonin dosage) and to detect resistance-related traits as soon as possible [10]. This last segment of the laboratory’s contribution to antimicrobial stewardship allows for a narrower empirical antimicrobial spectrum and the de-escalation (i.e. the reduction of the antimicrobial spectrum) of the ultimate targeted therapy. Furthermore, the rapid detection of a resistant trait may allow us to upgrade the antibiotic strategy in case of inadequate empirical therapy. At present, this detection is a hot topic and a top priority for clinical microbiologists [6]. Rapid detection tests clearly belong to this armamentarium.

1.3.2. The key role of β -lactam antibiotics in antimicrobial chemotherapy

According to their intrinsic properties, such as bactericidal activity, low toxicity and few side effects, a wide spectrum of activity, excellent pharmacokinetic parameters, and relatively low price, β -lactams are among the most frequently prescribed antimicrobial agents, ranking first in human medicine and second in veterinary medicine [11,12]. The fact that they are prescribed for treating community-acquired infections as well as severe nosocomial infections is due to their wide spectrum of antibacterial activity.

1.3.3. The evolving world of β -lactamases in *Enterobacteriaceae*: an endless race?

The diversity of β -lactamases identified in *Enterobacteriaceae* is high in contrast to the diversity of clinically relevant,

multidrug-resistant gram-positive bacteria such as vancomycin-resistant *Enterococci* (VRE) or methicillin-resistant *Staphylococcus aureus* (MRSA) in which the variety of resistant genes responsible for key antimicrobial compounds (i.e. β -lactams or glycopeptides) is limited, including one or two genes (*mecA/C*, *vanA/B*) [4]. Regarding *Staphylococci*, it is notable that a significant percentage of the clinical strains (40–80%) produce penicillinase, of which different types have been described. The Ambler scheme classifies β -lactamases into four classes (A, B, C, and D) according to the protein homology of enzymes. Considering the current challenges in antimicrobial therapy and infection control, there will be a focus on the two families of β -lactamases found in *Enterobacteriaceae*, the class A extended-spectrum β -lactamases (ESBL) and the carbapenemases that gather enzymes from the class A, B, and D groups [4]. Plasmid-encoded AmpC-type β -lactamases belong to Ambler class C and are less relevant than ESBL and carbapenemase in terms of public health. Nevertheless, this class should be monitored with caution. ESBLs inactivate the primary first-line therapy for gram-negative bacteria, namely the expanded cephalosporins (cefotaxime, ceftriaxone, ceftazidime, and cefepime). The most commonly identified ESBLs in *Enterobacteriaceae* to date are the CTX-M enzymes, followed by the TEM and SHV derivatives. Carbapenemases inactivate carbapenems, and they may either possess a serine residue in their active site (class A and class D β -lactamases) or require zinc ions in their active sites to be functional (class B β -lactamases, which are also named metallo- β -lactamases). The most commonly identified carbapenemases in *Enterobacteriaceae* are the class A β -lactamase KPC, the class B IMP-, VIM-, and NDM- enzymes, and the class D OXA-48-like enzymes. The biochemical diversity of carbapenemases leads to a degree of variability in the hydrolysis profiles; it constitutes an additional challenge for their detection. The hydrolysis and inactivation of carbapenems, which are the primary last-resort antimicrobial compounds, constitute a real threat to public health [13].

1.3.4. Impact of β -lactam resistance detection on clinical success: the individual point of view

Resistance to β -lactams is a risk factor for therapeutic failure, and, for some special situations, it is a risk factor for death. With regard to empirical therapy, the delayed administration of an effective therapy has a negative effect on the clinical outcome such as the clinical cure, length of hospitalization, or, for the most severe infections or the weakest patients, the survival rate [14]. This relationship is clearly established particularly for septic shock. Considering the definitive therapeutic option, resistance to β -lactams is associated with poor outcome, with infections caused by ESBL- or carbapenemase-producing strains associated with a higher mortality rate. For example, the mortality caused by *Klebsiella pneumoniae* bacteraemia is significantly increased if the given strains are resistant to carbapenems [15,16].

1.3.5. Impact of MDRO detection on infection control policy success: the collective point of view

The efficacy of the ‘search and destroy’ policy is strongly associated with the rapid implementation of the appropriate

infection control measures such as patient isolation and dedicated health-care staff [17]. In 2014, Fournier and colleagues showed that the implementation of extensive infection control measures during the first 48 h of the hospitalization of an MDRO carrier is statistically associated with fewer secondary cases compared to the results of delayed strategies [18]. The multiplicity of patients requiring isolation measures is associated with decreased hygiene compliance [19].

1.4. Practical aspects of the topic

The rapid detection of antibiotic resistance can be considered at the different steps of the microbiological diagnosis process, either directly from clinical specimens or after a primary culture step (from broth cultures such as blood cultures or agar-based cultures), from carriers or infected patients. The putative contribution of these tests is summarized in Table 1.

Some issues that are discussed later could be extended to the management of other MDROs, including gram-positive bacteria (VRE and MRSA) and non-fermentative *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Nevertheless, the impact of the rapid detection of those two bacteria at the species level and in terms of resistance traits is primarily limited to intensive care units. Beyond the focus on a single antibiotic class, i.e. the β -lactams, we targeted a particular resistance mechanism, which is the production of β -lactamases, the enzymes that inactivate the β -lactam ring that is responsible for antimicrobial activity. Along with the production of β -lactamases, additional mechanisms have been described to be responsible for β -lactam resistance such as efflux pumps, penicillin-binding protein modifications, and the decreased production or loss of porins. However, the most significant mechanism of β -lactam resistance either clinically or epidemiologically in *Enterobacteriaceae* remains the production of β -lactamases. Since the genes that encode β -lactamases might be transferable, facilitating their detection to improve their control is of paramount importance for controlling their spread. Among the very large panel of β -lactam compounds that includes penicillin, cephalosporins, monobactams, and carbapenems, we focused on two categories of

antibiotics, the broad-spectrum third-generation cephalosporins (3GC) and the carbapenems. Due to their low cost, great clinical efficiency, and global availability inside and outside of hospitals, the 3GC constitutes a first-line choice for empirical and definitive therapy in many cases. However, carbapenems constitute last-resort options for which the clinical efficiency and tolerability are higher than that of alternative anti-biotherapies such as polymyxins or tigecycline.

2. Biochemical diagnostics for rapid detection of antibiotic-resistant enterobacteriaceae

2.1. What is the gold standard for the biochemical detection of β -lactam-resistant *Enterobacteriaceae*?

The definition of the gold standard in antimicrobial testing for clinical purposes is one of the most important issues for microbiologists and clinicians. From a biochemical point of view, the gold standard for the biochemical characterization of a β -lactamase requires the lysis of the cell, the purification of the proteins, the analytic isoelectric focusing of the extract, and the identification of its hydrolytic activity using a penicillin such as Nitrocefim® (Oxoid, Hampshire, U.K.). Beyond this basic characterization, the hydrolytic activity spectrum should be measured using an UV spectrophotometer against a panel of β -lactam compounds and inhibitors; the specific activity and kinetics parameters could then be established. Clearly, this method may take days and may not be implemented as part of a clinical approach that requires prompt results to optimize the therapeutic options.

2.2. Recent advances in biochemical diagnostics for the rapid detection of antibiotic-resistant *Enterobacteriaceae*

The identification of broad-spectrum β -lactamase activity is the cornerstone of the biochemical approach to rapidly detecting broad-spectrum β -lactam resistance. Due to the high diversity and prevalence of β -lactamases among *Enterobacteriaceae*, the biochemical approach should be more selective, focusing on ESBL and carbapenemase produc-

Table 1. Key challenges in antimicrobial chemotherapy and putative contribution of the rapid diagnostic techniques in clinical laboratory.

Key challenge		Responses
At the individual level	At the community level	
-To identify the carriers of bacteria resistant to antibiotics	-To rapidly identify the carriers of MDRO ^a and to prevent cross transmission by implementing isolation measures	-To identify risk factors for resistant bacteria carriage as history of antibiotic consumption or hospitalization -To identify history of MDRO carriage -To detect MDRO carriage using selective culture-based approach and to confirm the presence of resistance traits
-To identify resistant traits among bacteria responsible for sepsis	-To limit the use of empiric broad range antimicrobial therapy	-To detect resistance traits directly from clinical specimens -To perform culture-based diagnosis of bacteria responsible for sepsis and to perform antimicrobial susceptibility testing/to detect resistant traits from cultivated bacteria
-To avoid extensive impact onto the natural microbiota, especially the intestinal microbiota, that promote the acquisition of <i>Clostridium difficile</i> or drug-resistant bacteria		-To detect resistance traits directly from biological specimen

^aMultidrug-resistant organisms.

tion. Two approaches have been developed for this detection, the colorimetric approach and the use of **matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF)** technology.

2.2.1. The colorimetric approach

The colorimetric approach consists in obtaining a variation in the color of the reagent medium resulting from a hydrolytic activity that modifies the chemical composition of the medium. This variation could be detected by eye or it could be measured by a spectrophotometer.

2.2.1.1. The use of a chromogenic substrate to detect the β -lactam hydrolysis capacity of bacteria.

The principle of this approach is to specifically select a substrate for the targeted enzymes; the hydrolysis of this enzyme would lead to the production of a product that shows a different color relative to the initial color of the substrate. The ancestral example of this approach was the Cefinase® test that was marketed in the early 1970s to detect the penicillinase of *S. aureus*. Nitrocefin (Oxoid, Hampshire, U.K.) was reported in 1972 as a chromogenic cephalosporin that acted as a substrate for any type of β -lactamases [20]. This substrate cannot differentiate between narrow- and extended-spectrum β -lactamases (ESBLs) in *Enterobacteriaceae*. Some authors have attempted to improve this test by adding β -lactamase-specific inhibitors and optical density measurements (i.e. the Pentawell test), but neither ESBLs nor carbapenemases could be accurately identified [21].

2.2.1.1.1. The detection of ESBL activity. The β -Lacta test® (Bio-Rad, Marnes-La-Coquette, France) is the first commercial kit for using this approach to detect ESBL-producing *Enterobacteriaceae* [22]. It consists of a chromogenic substrate (HMRZ-86) that was discovered more than 10 years ago. This substrate resists narrow-spectrum β -lactamases but is hydrolyzed in the presence of an ESBL, yielding a red product; an orange color should be considered an equivocal, undetermined result (Figure 1) [23]. The β -Lacta test® was first used with isolated colonies and was then conducted directly from clinical specimens such as urine or blood cultures [24–26]. The characteristics and performance of this test are reported in Table 2. The β -Lacta test® was initially developed to detect all

the broad-spectrum β -lactamases that hydrolyze cephalosporins. This test not only detects ESBLs but also some hyperproduced cephalosporinases and some carbapenemases with activity toward expanded-spectrum cephalosporins [27]. The β -Lacta test® may be performed in parallel with a rapid identification method, such as MALDI-TOF MS [24,25]. Although the sensitivity of this test for detecting ESBL-producing *Enterobacteriaceae* is high, the positive predictive value is rather low because this test also detects AmpC hyperproducers, the class A carbapenemase KPC, the OXA-48-like class D carbapenemases, and, at a lower efficacy, the class B carbapenemase producers [27].

2.2.1.1.2. Detection of carbapenemase activity. The β -CARBA test® (Bio-Rad, Marnes-La-Coquette, France) has recently been marketed for the detection of carbapenemase among freshly isolated *Enterobacteriaceae* colonies; it employs a chromogenic substrate (<http://www.bio-rad.com/fr-fr/sku/68260-beta-carba-test>). The color change in the reactive medium must be visualized by eye within 30 min. The bacterial culture must be fresh (<24 h). The manufacturer of the β -CARBA test® reports a sensitivity of 100% (207/207) when testing colonies that were obtained on Columbia agar containing 5% sheep blood, and they also report a specificity of 97.8% (89/81). The sensitivity decreases significantly if the colonies are picked from a chromogenic (98.6%) or Drigalski (77.6%) medium. The false-positive non-carbapenemase-producing strains were AmpC producers. Recently, Compain et al. tested the β -CARBA test® against a collection of 42 carbapenem-resistant *Enterobacteriaceae*, with 30 out of 42 being carbapenemase producers [29]. Within the 30 min of incubation recommended by the manufacturer, the test was positive for 26 of the carbapenemase-producing strains, missing four OXA-48-producing *E. coli*; these missed strains yielded a positive test result if the incubation was prolonged to 1 h. No false-positive results were obtained. Since only a single published study is available, those data will be treated with caution, and they require further clinical validation.

2.2.1.2. The use of a non-chromogenic substrate in which hydrolysis leads to a color change in the medium

2.2.1.2.1. Detection of ESBLs. The newly marketed Rapid ESBL NP Test® (Senova GmbH, Weimar, Germany) is an

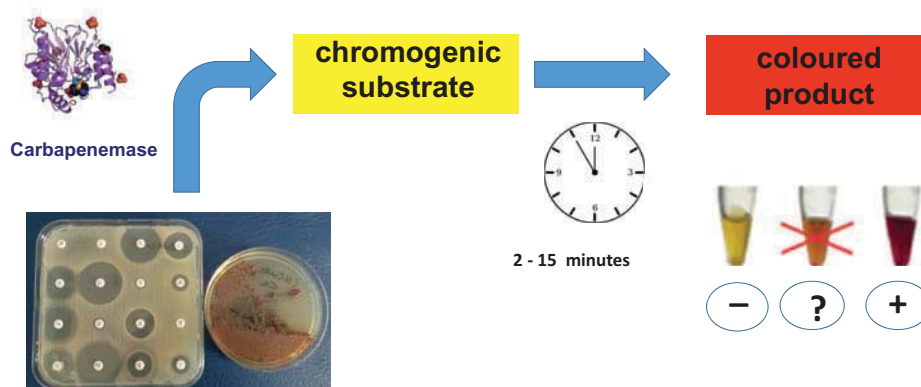


Figure 1. The β -lacta Test®.

Table 2. Principle and performances of rapid biochemical tests for the detection of clinically relevant β -lactam-resistant *Enterobacteriaceae*.

Principle/name of the test	Targeted enzymes	Required additional supplies	Delay for first/definitive results	Performances on cultured bacteria	Performances on clinical specimen	References
<i>Colorimetric–chromogenic substrate</i>						
β -Lacta test®	ESBL ^b	None	15 min	Sensitivity: 88% Specificity: 71%	Urines: sensitivity: 94%, specificity:100% (positive blood culture: sensitivity: 95.7%, specificity: 100%)	[22–28]
β -CARBA test®	Carbapenemase	None	30 min	No direct comparison: sensitivity 87%, specificity 100%		[29]
<i>Colorimetric–non-chromogenic substrate</i>						
Rapid ESBL NP test®	ESBL ^b	None	20 min	Sensitivity: 95% Specificity: 100%	Urines: sensitivity 98%, specificity 99.8%, positive blood culture: sensitivity 100%, specificity 100%	[28,30–32]
Rapid ESBL Screen kit®	ESBL ^b	None	30 min/2 h	Sensitivity: 92% Specificity: 83%		[28]
Rapidec® Carba NP test	Carbapenemase	None	30 min/2 h	Sensitivity: 99% Specificity: 100%	Positive blood culture: preliminary experimental data	[33]
Rapid CARB Screen®	Carbapenemase	None	5 min/2 h	Sensitivity: 89.5% Specificity: 70.9%		[33]
Rapid Carb Blue kit®	Carbapenemase	None	15 min/1 h	No direct comparison: sensitivity 100%, specificity 100%		[34]
<i>Mass spectrometry detection</i>						
Pending marketed kits from MALDI-TOF MS ^a platforms manufacturers	Carbapenemase	MALDI-TOF MS ^a platforms	1–4 h	Sensitivity: 72.5%–100% Specificity:98%–100%	Positive blood culture: preliminary experimental data	

^aMatrix-assisted laser desorption ionization-time of flight mass spectrometry.

^bExtended-spectrum β -lactamase.

^cNonevaluated.

Indications in brackets are not validated by the manufacturers.

Performances in bold **are** provide from direct comparisons (as the corresponding reference).

upgraded version of the ESBL NDP test that was first developed as a home-made test for detecting the hydrolysis of cefotaxime (and any other broad-spectrum cephalosporins) in less than 20 min by using a pH indicator (phenol red) [30]. In comparison to a negative control well without antibiotic, the reactive well containing cefotaxime and the pH indicator experiences a color change from red to yellow if ESBL produces some carboxyl-acid groups resulting from cefotaxime hydrolysis. The same reaction occurs in the presence of a penicillinase inhibitor, namely tazobactam, which inhibits the hydrolysis reaction, thereby helping to identify the ESBL nature of the β -lactamase (Figure 2). The performance of this home-made test has been evaluated with either cultured bacteria (sensitivity, 92.6%; specificity, 100%) or directly from urine (sensitivity, 98%; specificity, 99.8%) or positive blood culture samples (sensitivity, 100%; specificity, 100%). Its sensitivity is excellent, particularly for detecting CTX-M producers (100%) [30–32]. A copy of the ESBL NDP test has been marketed (the Rapid ESBL Screen kit®, Rosco-Diagnostica, Tastrup, Denmark); neither the specific characteristics nor performance results are published. A performance comparison of three tests for detecting the ESBL-producing bacteria (the rapid ESBL NDP test, the Rapid ESBL Screen kit®, and the β -Lacta test®) was recently performed using the exact same collection of strains [28]. The ESBL NDP test reached high sensitivity and specificity (95% and 100%, respectively). The limits of the β -Lacta test® in selectively detecting the ESBL-producing bacteria among cephalosporin-resistant

Enterobacteriaceae were again highlighted here (specificity, 71%). Regarding the Rapid ESBL Screen kit®, the protocol appeared to be time-consuming (2 h) and the results varied considerably according to the reading time. The sensitivity and specificity of the Rapid ESBL Screen kit were lower than those of the ESBL DP test.

2.2.1.2.2. Carbapenemase detection. The first commercial test was the RAPIDEC® Carba NP test (bioMérieux, La Balmeles-Grottes, France). The marketed version and its prototypes (see later) are the most frequently evaluated methods for the rapid detection of carbapenemase activity, with more than 60 published references, primarily in highly rated journals. This test is an identical version of the original Carba NP test that took advantage of several improvements. Notably, the Carba NP test is now recommended as a first-line test for screening carbapenemase activity by the Clinical and Laboratory Standards Institute in the US [35]. This test targets any type of carbapenemase activity by detecting the hydrolysis of imipenem. Originally, the Carba NP test was developed as an in-house technique using imipenem as substrate, with the principle of the test based on the acidification of the reaction medium when the β -lactam ring of the imipenem molecule is opened because of the hydrolytic activity of carbapenemases [36]. The modification of the pH of the reacting medium is revealed by a color shift in the pH indicator, namely the phenol red that turns from red to yellow with acidification (Figure 3). Another version of the in-house Carba NP test, the Carba NP II test, has been developed, and it includes

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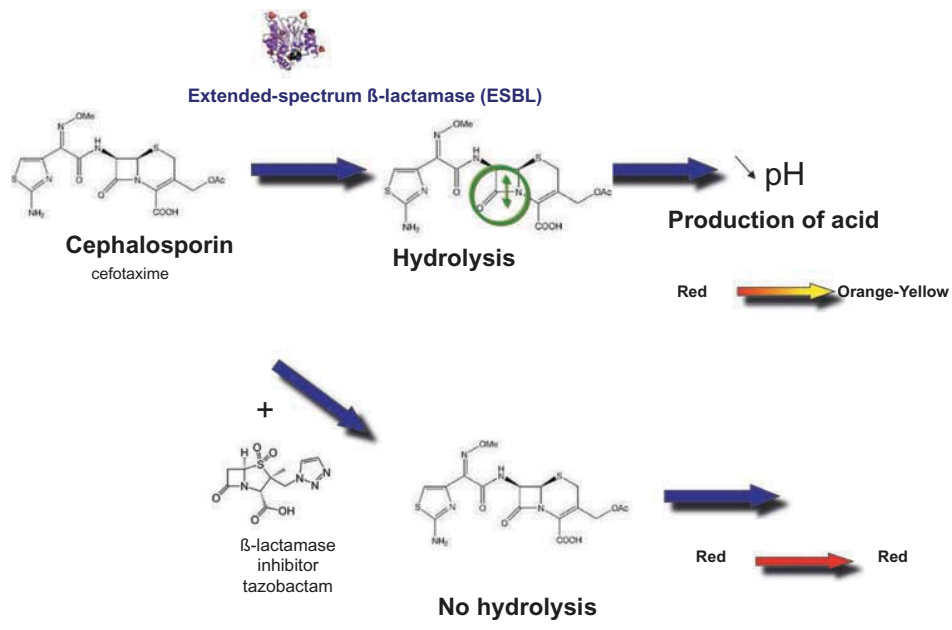


Figure 2. The ESBL NP ©Test.

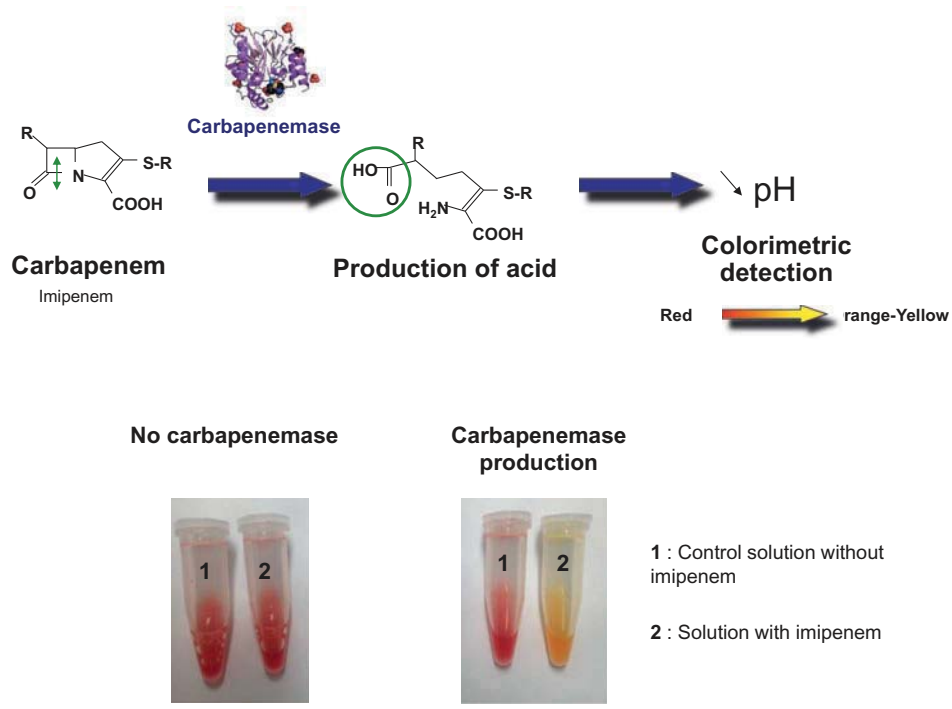


Figure 3. The Carba NP ©Test.

additional wells with class-specific β -lactamase inhibitors [37,38]. This version allows investigators to screen and to more precisely characterize the carbapenemase activity of a given strain, allowing them to differentiate between enzymes for which the activity is inhibited by clavulanic acid (class A), by EDTA (class B), or by neither of those two (class D). To date, the Carba NP II test has not been developed industrially. Interestingly, the hydrolytic activity of metallo-carbapenemases is boosted by reagent medium that includes zinc, a

basic element that is essential for metallo- β -lactamases. The global use of the Carba NP test allowed for some improvement of its performance, either for the recommended inoculum particularly for mucoid strains, the nature of the imipenem, or the type of agar medium from which tested colonies are picked [39–43]. The sensitivity of the carbapenemase detection could be negatively influenced by several culture media such as the Drigalski or MacConkey agar plates, considering that colored pigments may interfere with the

reading of the test [44]. This in-house method has been improved several times and then industrially developed, and it has been on the market since early 2015 under the trade name RAPIDEC® Carba NP test (Figure 4) [45–50].

The ability of the RAPIDEC® Carba NP test to detect carbapenemase activity depends at least in part on the level of enzyme production and the enzyme's capacity to hydrolyze the substrate and the tested inoculum (see later). The performance of the RAPIDEC® Carba NP makes this biochemical test appropriate for the rapid (30 min to 2 h) and convenient screening of carbapenemase activity in gram-negative bacteria. Its sensitivity and specificity reached 96% in the original study, subsequently increasing to 97.8% and 98.5%, respectively, during independent evaluations [45–47]. Some publications reported relatively low sensitivities for in-house and RAPIDEC® Carba NP tests for detecting the OXA-48-type producers [42,49,50]. Those results may be explained by the weaker carbapenemase activity of the OXA-48-like carbapenemases, or the use of old cultures or too little inoculum. Notably, the use of the correct inoculum (one full 10- μ l loop for the Carba NP test) and a proper culture plate are critical for obtaining good sensitivity. The zinc concentration and/or the color of the colonies on chromogenic medium must be optimal for detecting the enzymatic activity with enough sensitivity [49]. Since the CarbaNP test® was successfully challenged directly against positive blood cultures, ongoing studies are evaluating the performance of the RAPIDEC® Carba NP directly from clinical specimens (Pr Nordmann, personal communication) [51].

Commercial alternatives to the RAPIDEC Carba NP test have been developed to screen for carbapenemase activity, such as the Neo-CARB kit®, which was formerly the Rapid CARB Screen® or the Rapid Carb Blue kit® (Rosco Diagnostica A/S, Taastrup, Denmark). The former is a copy of the RAPIDEC® Carba NP and the latter test is identical to the RAPIDEC® Carba NP, with the exception of the color indicator, which is bromothymol blue instead of phenol red [34,39,52–54]. Furthermore, and beyond the performance of these tests in the original publications, their direct comparisons against the Carba NP test and/or the RAPIDEC® Carba NP showed superiority in terms of performance (Table 2) [33,55–57].

2.2.1.3. Advantages of the colorimetric approach. The colorimetric approach fulfills the requirements of an optimal test for β -lactamase detection in that it is reliable, rapid, cheap, and requires no or very limited additional supplies. The biochemical detection of ESBL or carbapenemase activities permits researchers to identify any type of enzyme, whereas molecular-based techniques only detect the genes that are included in the corresponding test.

One primary advantage of colorimetric approaches is that they can be applied directly to colonies that are grown on selective media for the rapid detection of multidrug-resistant strains. For the detection of either ESBL- or carbapenemase-producing bacteria, the ESBL NDP and the Carba NP tests are perfectly suitable. The screening of MDRO carriers can therefore be optimized in terms of effectiveness, combining excellent sensitivity and rapidity, which are two critical features in infection control policy.

2.2.1.4. Pitfalls of the colorimetric approach. The expected performance of colorimetric methods is based on their capacity to detect the hydrolysis activity of a broad range of biochemically diverse enzymes (and therefore it has high sensitivity) without amplifying nonspecific activities (therefore providing highly specific results). The sensitivity of a biochemical test with the aim of detecting an enzymatically mediated mechanism of antibiotic resistance depends on different factors such as (i) the amount of enzyme produced, which relies on the level of expression of the corresponding gene and the bacterial inoculum; (ii) the ability of the enzyme to hydrolyze the substrate, which relies on the extraction protocol; (iii) the affinity of the enzyme for the substrate, which relies on the pharmacokinetics properties of the enzyme/substrate couple; and (iv) the sensitivity of the revelation step, which relies on the indicator properties and the mode of measurement (by naked eye or photometric means).

In addition to the lack of sensitivity, phenotypic colorimetric tests might run into some interference from the undesirable hydrolytic activities of nontargeted enzymes. This result is found for intrinsic AmpC-type cephalosporinases from different enterobacterial species or the K1 penicillinase of *Klebsiella oxytoca*, which hydrolyzes broad-spectrum cephalosporins when they are overproduced (β -Lacta test®). Therefore, determining an accurate threshold for phenotypic colorimetric methods may be a critical issue. It is notable that the TAT of some assays could exceed 2 h. Additionally, those tests are not suitable for rectal screening due to the lack of specific recognition of their ESBLs or carbapenemases among all the microbiome bacterial activity.

2.2.2. MALDI-TOF MS approach

2.2.2.1. Principle of the MALDI-TOF MS approach. The implementation of MALDI-TOF MS technology in clinical laboratories has significantly modified bacterial diagnostics. Combined with the automation and implementation of a user-friendly interface, the identification of bacteria by mass spectrometry opens up a new area for microbiologists in terms of accuracy and TAT to deliver results [58]. This technology identifies the bacteria at the species and/or genus level from single isolated colonies on solid media, but it is also applied directly to positive blood and urine [58]. After a first rapid preparation step including sample application to a slide and the addition of an organic matrix solution, the identification can be performed in less than 1 min. If a clinical fluid or a positive blood culture is directly tested, a pre-processing step has to be performed. The MALDI-TOF MS principle is based on the ionization, with a laser source, of bacterial biomolecules containing proteins embedded in a matrix. Once they are ionized and sublimated into a gas phase, these molecules are accelerated into an electric field and projected onto a detector. They can then be separated and analyzed according to their time of flight. This delay between the ionization process and the final impact depends on their mass-to-charge ratio.

Commercial MALDI-TOF technology focuses on the identification of proteins or DNA molecules [58]. Several systems are on the market including different machines and databases; the

three primary commercial products are the Bruker Biotyper® (Bruker Daltonics, Wissenbourg, France), the bioMérieux Vitek MS System® (bioMérieux, Marcy l'Étoile, France), and the Andromas® (Beckman Coulter, Villepinte, France) system, the database of which can be implemented in different machines. Before bacterial identification using libraries of protein profiles via the MALDI-TOF concept, MS technology was developed to identify chemical compounds, including antibiotics. When coupled with liquid or gas chromatography, MS is the reference method for detecting and dosing antibiotics in clinical samples. Due to its cost and lack of user friendliness, this approach was replaced by immunological techniques such as ELISA. Nevertheless, MS maintains the capacity to detect antimicrobial molecules and, interestingly in the context of β -lactam resistance, the products of the antibiotic after hydrolysis. Therefore, MALDI-TOF MS has great potential for the detection of resistance traits among bacteria strains in two particular contexts, i.e. (i) the detection of the enzyme and (ii) the detection of a substrate degradation replaced by the signal of its product after a variable incubation time.

When dealing with β -lactamases, the identification of a discriminatory peak corresponding to a protein with significant hydrolysis activity was first considered, but it was rapidly abandoned in favor of observing the degradation product [58]. MALDI-TOF MS is used to identify both the β -lactam molecule and its degradation product. As an example, the hydrolysis of imipenem is interpreted as the disappearance/appearance of specific peaks (300 Da and 254 Da, respectively) when this antibiotic is added to a bacterial suspension, and the MS spectrum is measured at the time of substrate addition and after 20 min of incubation. The TAT of this process is approximately 30 min [59]. Some other protocols are more time-consuming, requiring an incubation time from 1 to 4 h [60–62]. The performance of this approach depends on the metabolite/imipenem ratio but also the metabolite cut-off. For example, in Knox et al., the MALDI-TOF MS approach was compared to the Carba NP test; the breakpoint was determined to be a disappearance of 95% of the area under the curve for the test compared to the control replicate [60]. In this study, the performance of the two methods was equivalent. Beyond the various published home-made protocols with differences in the substrate (meropenem/imipenem/ertapenem/faropenem), the lysis conditions, the incubation time, and the parameters/breakpoints, a specific module should be marketed in the very near future (MALDI Biotyper STAR BL®, Bruker Daltonics, Wissenbourg, France) [63].

Additionally, the chemical properties of the enzyme may be indirectly investigated by adding specific inhibitors (such as the β -lactamase inhibitor clavulanic acid) that hinder the β -lactamase hydrolytic activity and therefore slow the disappearance of the β -lactam native peak. Several companies have developed kits for that purpose, and they have produced protocols that are about to be marketed. Some authors have attempted to adapt the protocol to positive blood cultures, and it is much more time-consuming (3–4 h) since a pre-culture is needed to amplify the amount of bacteria in the specimen [64,65]. Next to β -lactamase production, the loss of a porin that allows the entry of the antibiotic inside the bacterial cell constitutes a clinically and epidemiologically relevant

mechanism of resistance in *Enterobacteriaceae*. The MALDI-TOF MS technology could replace cumbersome and time-consuming reference methods such as sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which is laborious to use routinely. For example, the loss of porins (OmpK35, OmpK36) from *Klebsiella* species may lead to carbapenem resistance. The SDS-PAGE technique is the reference method for identifying the presence/absence of these porins [66]. After the identification of the corresponding peaks, MALDI-TOF MS permits the localization of these porins and objectivizes the loss of OmpK36 [66]. As previously reported, the infection control consequences and particularly the risk of an epidemic spread of the genetic support underlying a resistance trait are associated with its mobility and its capacity to transfer from one bacterium to another. In the present case, the genetic trait is the truncation of the chromosomal gene that cannot be horizontally transferred. Although it is technically reliable, this potential application has not been included in the panel of MALDI-TOF MS kits that are about to be marketed.

2.2.2.2. Advantages of the MALDI-TOF MS approach. The hydrolytic activity is determined regardless of the genetic basis of the resistance and the type of β -lactamase. The sensitivity of this technology seems to be good regardless of the nature of the β -lactamase, including for OXA-48-like enzymes [67]. The theoretical cost in consumables for this approach is low (less than 0.1 euro, excluding the cost of acquiring the machines that are now very common in clinical laboratories) as is the cost associated with technician time. To date, the real cost of the commercial kits remains unknown.

2.2.2.3. Pitfalls of the MALDI-TOF MS approach. A direct comparison of the MALDI-TOF MS and Carba NP test results highlights some discrepancies. For unresolved reasons, several NDM- or VIM-producing *Proteus* spp. and *Morganella* spp. are not detected by the MALDI-TOF MS approach [60]. The authors suggested that there was a problem related to enzyme availability for the MALDI-TOF method, which did not include a cell lysis step. Another explanation could be the type of carbapenem compound under testing. In fact, no false-negative result was obtained in the two other studies including *M. morgani* and *Providencia* sp. strains, but they tested the degradation of ertapenem or meropenem instead of imipenem [61,62]. Moreover, for some OXA-48 variants (OXA-204) that exhibited a very low increase in the carbapenem minimal inhibitory concentrations (MIC), a false-negative result could be found [59]. However, the hyperproduction of cephalosporinase could lead to a false-positive result [59]. An additional complex spectral comparison did not improve the performance of the challenged protocol. The standardization of the experimental conditions through the marketing of kits may help to solve these problems. Notably, a positive signal when using the MALDI-TOF MS that indicates β -lactamase activity cannot be translated into an estimation of a MIC that might still be below the resistance breakpoint. In other words, extrapolating whether the strain is resistant to the tested antibiotic is not possible. However, since infection control guidelines are based on the presence of certain enzymes (e.g. ESBL or carbapenemases) and preferably but not systematically to the exact MIC value, this approach makes clinical sense. Finally, if

using MALDI-TOF MS technology directly on positive blood cultures, the same problems arise as the problems of using it for identification purposes, including decreased sensitivity and time-consuming pretreatment requirements. To date, using those types of techniques for detecting multidrug-resistant strains within a mixed flora (gut) remains impossible.

3. Antigenic rapid detection of antibiotic-resistant *Enterobacteriaceae*

Recently, a new category of test was marketed on the basis of the immunological detection of some specific β -lactamases as the KPC and OXA-48 enzymes [68–70]. The performance of these immunochromatographic tests (OXA-48/KPC K-set®, Coris BioConcept, Gembloux, Belgium) that are easy to perform and to interpret seems to be higher. This lateral flow immunochromatographic assay uses monoclonal antibodies and colloidal gold nanoparticles bound to a nitrocellulose membrane, and it provides results from a single colony in 15 min. Its performance reaches 100% specificity and sensitivity, with a limit of detection of 10^6 CFU/ml [69]. A recent study that employed the whole genome sequencing approach as the reference method showed the complete concordance between those two methods [70]. To the best of our knowledge, no corresponding assay exists for the detection of ESBLs, and the high diversity of enzymes that must be detected likely prevents further developments.

3.1. Advantages of the immunological approach

Theoretically, the immunological approach could be as sensitive and specific as the enzymatic approach. In fact, both methods depend on the level of protein production, with the immunological approach revealing the presence of this protein and the enzymatic one revealing its biochemical activity. In published studies, these two approaches were strictly equivalent for detecting *bla*_{KPC} genes [71]; with regard to *bla*_{OXA-48} carbapenemases, the immunological method detected a few additional cases that were missed or that yielded equivocal results with the enzymatic method (3 out of 100 in the study by Dortet et al., 7 out of 130 in Glupczynski et al.) [71,72]. The OXA-48 variants that do not possess any significant carbapenemase activity such as OXA-163 and OXA-405 have been reported by the manufacturers as nonreacting variants. The small number of clinical strains ($n = 3$) producing those types of enzymes that were tested in three published studies unfortunately prevent any valid conclusions. This high level of specificity among the large number of OXA-48 variants is clearly a positive aspect of the immunological approach. Some authors have tested the OXA-48K-set® directly from spiked blood cultures (Aerobic FA Plus 30-ml bottles, BacT/Alert system, bioMérieux, Durham, supplemented with 10 ml of heparinized horse blood). The testing of a lysate after an 18-h incubation time yielded excellent results [69].

3.2. Drawbacks of the immunological approach

Targeting a specific type of enzyme alone as the first step is an approach that cannot be used as a screening test. Its price (ca.

10€ depending on the country) and the narrow panel of available targets limit its use as a first-line strategy. It could be of interest only in some specific settings, or in countries with a high predominance of a single type of carbapenemase, or during the course of an investigation of an outbreak context that generates secondary cases that are presumably related to strains that produce well-identified carbapenemases. Beyond the remaining doubts about the functionality of detecting specific proteins that have been extensively acknowledged, we must be aware of the risk of emerging specific enzyme variants for which the targeted antigenic site might be modified, and they consequently would not be detected by the initially designed antibody. Regarding the continuously increasing number of variants, a thorough monitoring of the performance of these tests must be completed. The most recent data are quite reassuring: all the OXA-48 variants with carbapenemase activity, such as OXA-181, OXA-204, OXA-232, OXA-244, OXA-245, OXA-436, and OXA-484, were successfully detected [70]. Nevertheless, the OXA-48K-set® detected the naturally occurring OXA-48-like enzyme from *Shewanella* spp., which is a shortcoming [70,73]. Although the prevalence of this bacterial genus in human specimens seems to be rare, we support the presumptive identification of suspected colonies with an oxidase test (*Shewanella* spp. as an oxidase-positive bacterium as opposed to *Enterobacteriaceae*) or a MALDI-TOF identification. Due to the difference in terms of the infection control measures that should be implemented, a positive result should be interpreted according to an unambiguous genus identification. Notably, this technique cannot be used directly from clinical specimens. In conclusion, the ICT method could be of interest for detecting carbapenemase from isolated colonies in some specific settings when the two targeted enzymes are highly prevalent among carbapenemase-producing strains.

4. Recent advances in molecular diagnostics for the rapid detection of antibiotic-resistant *Enterobacteriaceae*

For two decades, molecular techniques have taken up an increasing and now prominent place in clinical laboratories. The primary technologies that are now implemented as routine methods are **polymerase chain reaction (PCR)**, including real-time PCR for the detection of pathogens and their associated virulence or resistance genes, PCR hybridization, including the microarray, to identify molecular sequences, and sequencing (in particular, the so-called ‘next generation sequencing’ (NGS)) that will likely be considered as routine techniques within a few years. Numerous reviews have been published recently about the contribution of nonphenotypic and/or molecular tests.

4.1. The DNA-targeted PCR-based approach

The PCR-based approach was the earliest molecular method to be implemented in clinical laboratories to detect β -lactam resistance genes among *Enterobacteriaceae*. Single end-point PCRs were first described in combination with a confirmation step using the enzymatic restriction of amplified fragments

and/or size evaluation by agar electrophoresis, or DNA–DNA hybridization. This multiplex process was laborious and was subject to contamination at each step in the process. Further improvements were implemented, such as the multiplexing and miniaturization of the hybridization step. Real-time PCR permitted the combination of the amplification and detection steps in a single step, limiting the risks of environmental contamination by amplicons. Home-made PCRs were progressively replaced by commercial kits including sound protocols and quality controls. A summary of the primary commercially available kits is reported in Table 3. After specific multiplex PCR amplification, the amplified DNA fragments are revealed by hybridization with a panel of specific probes. The support of this hybridization could be the reactive medium when using real-time PCR or a solid surface. The number of probes actually increases in parallel with the miniaturization of the support, as with the microarray. The detection of resistance genes could be performed independently, or it could be included in multiplexed panels for the diagnosis of infections, for example, in the context of bloodstream or respiratory infections.

4.1.1. The whole automated real-time PCR platform: the example of the Xpert® system

The Xpert® system is the first fully automated PCR instrument to allow for the implementation of a PCR diagnostic method in a very large panel of clinical laboratories. This technology requires neither technical competency nor skilled technologists, allowing its use over a broader period during open laboratory hours. This type of nucleic acid amplification test might be considered as a molecular diagnostic POCT. The different steps are performed in a single compact cassette after the introduction of the specimen, combining DNA extraction, amplification, and revelation. An internal positive control is included in the multiplexed targets. The Carba-R assay® (Cepheid, Sunnyvale, CA, USA) runs on the Gen Expert® platform, which is suitable for the detection and quantification of numerous bacterial or viral species, in addition to human tumor targets. This test is marketed to detect several carbapenemase genes directly from a rectal sample; a derived protocol is provided by the manufacturer to use this assay on cultured bacteria [95,96].

4.1.2. The Check-Direct assay®

The Check assays® (Checks points, Wageningen, The Netherlands) include a large panel of different multiplex real-time PCR kits using different probes, with one of them targeting an internal control. The DNA extraction step must be performed outside the real-time PCR platform. The number and type of targeted genes vary according to the kit, including narrow- and/or broad-spectrum β -lactamase genes (see Table 3). Different PCR platforms may be used. Although they are thought to be cumbersome, expensive, and time-consuming, the Check assays® are used as a reference technique to characterize β -lactamase genes from isolated colonies. Recently, a new multiplex PCR panel was developed to detect the four primary types of carbapenemase genes directly from rectal swabs, i.e. the Check-direct CPE®

[74,77,97]. Furthermore, successful attempts to shorten the duration of the DNA extraction step were recently published [74].

4.1.3. Other systems

Numerous commercial systems can detect and/or confirm the presence of resistance genes (see Table 3). They differ from one another in their theoretical principles and their panel of targeted genes. The most interesting features of those tests would be their potential to identify both the bacteria and their resistance traits as a whole, particularly in blood cultures. The primary representative of these ‘broad-range multiplex PCR panels’ are the Filmarray® (bioMérieux, Marcy l’Étoile, France), the Verigene® (Nanosphere, Northbrook, US), the ePlex® (GenMark Dx, Zug, Switzerland), the Hyplex® superBug ID (Amplex Diagnostics GmbH, Gars-Bahnhof, Germany), and the Unyvero® (Curetis AG, Holzgerlingen, Germany), with the two first panels being the most widely tested ones. Regarding the Xpert® system, these PCR panels do not require extended skills and could theoretically be used 24 h per day. Interestingly, some assays seem to be valuable for the detection of resistant genes in other clinical specimens of high interest to diagnose respiratory, urinary tract, or joint infections due to resistant bacteria [82,86,98]. Regarding bloodstream infections, some of these assays could be positive earlier in comparison to blood culture systems [83]. Beyond their analytical performance, the adequacy of the targeted gene panel for local epidemiology and their costs (approximately 150 euros per test) are still considered as a limitation to their development (see later).

Other assays have been developed to characterize the β -lactamase content of a given isolate to decrease the cost and improve the detection of multiple allele variants in a single isolate. The Luminex® (Luminex Corporation, Austin, TX, USA) technology is a well-established detection approach that relies on colored microsphere-based flow cytometry assays. This test allows for the detection of specific alleles, antibodies, or peptides, and it has been already marketed for other purposes such as HLA typing, seroprevalence studies, or the detection of a broad panel of microorganisms directly from clinical specimens such as stools. Recently, a Luminex xTAG® assay was developed to detect ESBLs, plasmid-mediated cephalosporinases, and carbapenemases [94]. The modular multiplex oligonucleotide ligation-PCR procedure allows for the detection of β -lactamase genes and their variants for less than 5 euros per sample and a TAT of 5 h. Regarding the first published study, the sensitivity and specificity are excellent (100% and 99.4%); the different variants of the same β -lactamase genes (as *bla*_{TEM}) present in the same isolate could be separated. The subtyping of the *bla*_{CTX-M} gene is also performed. Additional data are required, but this approach seems to be promising.

4.1.4. Performance

4.1.4.1. Sensitivity. The performance of the molecular methods, and particularly the commercial multiplexed PCR assays, must be considered in view of different clinical or technical settings. The most favorable setting is the characterization of

Table 3. Examples of commercial molecular tests, including targeted genes, material requirement, and turnaround times.

Test name (manufacturer)	Targeted genes	Specimen	Technology/material requirement/instrumentation	Turnaround time (manufacturer's specification)	Reference
Carba-R assay ^a (Cepheid, Sunnyvale, CA, USA)	<i>bla</i> _{KPC} , <i>bla</i> _{NDM} , <i>bla</i> _{VIM} , <i>bla</i> _{OXA-48} , <i>bla</i> _{IMP-1} -like new version of the assay includes <i>bla</i> _{OXA-181}	Rectal swab (colonies) ^a	One-step multiplex real-time PCR using specific probes – dedicated platform (GenExpert [®])	<1 h	[74,75,76]
Check-Direct CPE [®] assay (checks points; Wageningen, The Netherlands)	<i>bla</i> _{SHV} , <i>bla</i> _{TEM} , <i>bla</i> _{CTX-M} including ESBL variants, <i>bla</i> _{KPC} , <i>bla</i> _{CMV} , <i>bla</i> _{DHA} , <i>bla</i> _{ACT/MIR} , <i>bla</i> _{ACC} , <i>bla</i> _{NDM} , <i>bla</i> _{VIM} , <i>bla</i> _{IMP} , <i>bla</i> _{OXA-48} -like, <i>bla</i> _{GIM} , <i>bla</i> _{SPM} , <i>bla</i> _{GES} including carbapenemase variants, <i>bla</i> _{OXA-23} , <i>bla</i> _{OXA-40} and <i>bla</i> _{OXA-58} , <i>bla</i> _{BEL} , <i>bla</i> _{PER} and <i>bla</i> _{VEB}	Strains, stool (positive blood culture)	Two-step ligation-mediated multiplex real-time PCR amplification – micro array hybridization, a non-dedicated real-time PCR platform as the LightCycler [®] (Roche, Brussels, Belgium), the BD Max [®] (Becton Dickinson, Oxford, U.K.), or an Applied Biosystems [®] Real Time PCR. The performances of the test in distinguishing separately the different positive signals may vary according to the PCR platform	2–6 h	[74,75,77–79]
• Check-ESBL [®]					
• Check-KPC-ESBL [®]					
• Check-MDR CT101 [®]					
• Check-MDR 102 [®]					
• MDR Carba [®]					
• Check-MDR 103XL [®]					
• Check-Direct CPE assay [®]					
Verigene Gram-negative blood culture nucleic acid test (Nanosphere, Northbrook, IL, USA)	<i>bla</i> _{CTX-M} , <i>bla</i> _{IMP} , <i>bla</i> _{KPC} , <i>bla</i> _{NDM} , <i>bla</i> _{VIM} , <i>bla</i> _{OXA-23} , <i>bla</i> _{OXA-40} , <i>bla</i> _{OXA-48} , <i>bla</i> _{OXA-58}	Positive blood culture	Micro-array hybridization-dedicated platform (Verigene Processor SP [®] and Verigene Reader [®])	2–2.5 h	[80,81]
FilmArray[®] blood culture identification Panel (bioMérieux, Marcy l’Etoile, France)	<i>bla</i> _{KPC}	Positive blood culture (biofilms from prosthetic joint infection, sterile fluids injected in blood culture bottle)	Nested-PCR and melting curve analysis, dedicated platform	1 h	[80,82–85]
ePlex[®] Blood Culture Identification Gram Negative Panel (GenMark Dx, Zug, Switzerland)	<i>bla</i> _{CTX-M} , <i>bla</i> _{OXA} , <i>bla</i> _{KPC} , <i>bla</i> _{VIM} , <i>bla</i> _{IMP} , <i>bla</i> _{NDM}	Positive blood culture	Multiplexed nucleic acid amplification test and a electrochemical detection technology with a dedicated platform	90 min	Manufacturer data.
Unyvero HPN Pneumonia, i60 ITI Implant Tissue Infection, and BCU Blood Culture (Curetis, Holzgerlingen, Germany)	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{NDM} , <i>bla</i> _{VIM} , <i>bla</i> _{IMP} , <i>bla</i> _{KPC} , <i>bla</i> _{OXA-48} , <i>bla</i> _{CTX-M}	Respiratory specimen, sputum, lavage, tracheal aspirate, blood cultures, liquid samples (exudate, pus, etc.)	Two-step multiplex PCR – hybridization with a dedicated platform	4–5 h	No published study [86–88]
CarbDetect AS-2 Kit, Identibac AMR-ve assays (Alere Technologies GmbH, Jena, Germany) ^b	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{OXA} , <i>bla</i> _{CMV} , <i>bla</i> _{ACC} , <i>bla</i> _{PER} , <i>bla</i> _{LEN} , <i>bla</i> _{VEB} , <i>bla</i> _{NDM} , <i>bla</i> _{SPM} , <i>bla</i> _{NDM} , <i>bla</i> _{IMP} , <i>bla</i> _{VIM} , <i>bla</i> _{KPC} , <i>bla</i> _{BIC} , <i>bla</i> _{DIM} , <i>bla</i> _{GES} , <i>bla</i> _{GIM} , <i>bla</i> _{COB} , <i>bla</i> _{MIR} , <i>bla</i> _{SIM} , <i>bla</i> _{IND} , <i>bla</i> _{HHM} , <i>bla</i> _{PAM} , <i>bla</i> _{SH} , <i>bla</i> _{SMB} , <i>bla</i> _{SME} , <i>bla</i> _{TMB} , <i>bla</i> _{OXA-48} -like	Strains	Two-step multiplex PCR – microarray hybridization with a dedicated platform	5–8 h	[89]
Hyplex SuperBug ID assays (Amplex Diagnostics, Gars, Germany)	<i>bla</i> _{KPC} , <i>bla</i> _{IMP} , <i>bla</i> _{VIM} , <i>bla</i> _{NDM} , <i>bla</i> _{OXA-48} -like (including <i>bla</i> _{OXA-181} , <i>bla</i> _{OXA-162} , <i>bla</i> _{OXA-204} , and <i>bla</i> _{OXA-244})	Strains (positive blood culture)	2 steps multiplex PCR – Hybridization /ELISA	2.5 – 4 h	[90]
eazyplex[®] Superbug complete A kit (Amplex, Gießen, Germany)	<i>bla</i> _{KPC-2} to <i>KPC-15</i> , <i>bla</i> _{NDM-1} to <i>NDM-7</i> , <i>bla</i> _{VIM-1} to <i>VIM-37</i> , <i>bla</i> _{OXA-48} , <i>bla</i> _{CTX-M-1} and <i>CTX-M-9</i> . The new version of the assay includes <i>bla</i> _{OXA-181}	Strains, positive blood culture (urines)	Loop-mediated isothermal amplification technology and a non-dedicated real-time PCR platform as the Genie (Optigene, Horsham, UK)	<30 min	[75,91,92]
VAPChip[®] (Eppendorf Array Technologies, Namur, Belgium)	<i>bla</i> _{TEM} , <i>bla</i> _{SHV} , <i>bla</i> _{CTX-M} , <i>bla</i> _{OXA-23} , <i>bla</i> _{OXA-24/40} , <i>bla</i> _{OXA-48} , <i>bla</i> _{OXA-58} , <i>bla</i> _{KPC} , <i>bla</i> _{VIM} , <i>bla</i> _{IMP} , <i>bla</i> _{MIR-7.13} , <i>IMP-1-like (The test can identify most of the narrow- and extended-spectrum <i>bla</i>_{TEM} and <i>bla</i>_{SHV} genes)</i>	Respiratory samples	Real-time PCR and microarray detection, on a dedicated RAP-ID platform [®]	4 h	[93]
Luminex xTAG[®] assay (Luminex Corporation, Austin, TX, USA)	<i>bla</i> _{TEM} , <i>bla</i> _{CTX-M} , <i>bla</i> _{SHV} , <i>bla</i> _{PER} , <i>bla</i> _{LEN} , <i>bla</i> _{VEB} , <i>bla</i> _{BEL} , <i>bla</i> _{OXA} , <i>bla</i> _{GES} , <i>bla</i> _{CMV} , <i>bla</i> _{ACC} , <i>bla</i> _{OXA} , <i>bla</i> _{OX} , <i>bla</i> _{DHA} , <i>bla</i> _{MIR/ACT} , <i>bla</i> _{SIM} , <i>bla</i> _{NDM} , <i>bla</i> _{IMP} , <i>bla</i> _{VIM} , <i>bla</i> _{IMP} , <i>bla</i> _{KPC} , <i>bla</i> _{BIG} , <i>bla</i> _{DIM} , <i>bla</i> _{GES} , <i>bla</i> _{OXA-48} -like, <i>bla</i> _{OXA-23} -like, <i>bla</i> _{OXA-51} -like	Strains	Multiplex oligonucleotide ligation PCR and detection using colored microsphere-based flow cytometric assays on a dedicated platform that could be shared by different biological domains	5 h	[94]

^aThe use of the test on colonies was not supported by the CE Marketing but a protocol is provided by the supplier.

^bThe kit is unable to distinguish between ESBLs and non ESBL variant among *bla*_{TEM} and *bla*_{SHV}. Indications in brackets are not validated by the manufacturers.

the β -lactamase content of bacterial cultures, and the most difficult setting is the detection of resistance genes in a highly diverse and DNA-rich environment such as the gut flora.

4.1.4.1.1. Sensitivity with pure bacterial cultures. In this case, the sensitivity of the assay relies only on the size of the targeted gene panel and not on the quantity of available DNA material. According to the increasing number of targets, including in the most recent commercial kits, the performance is excellent. For example, the Check-MDR CT103 XL array is a new product that allows for the amplification and identification of a large panel of β -lactamases, including ESBLs, cephalosporinases, and carbapenemases. Its sensitivity against a sample of 223 strains was 100% [79]. Moreover, the commercial assays include an internal control that detects the presence of *Taq* polymerase inhibitors.

4.1.4.2. Sensitivity with clinical specimens

4.1.4.2.1. Clinical specimens with poor bacterial diversity (positive blood culture, urine). The sensitivity of β -lactamase gene detection directly from clinical specimens such as urines or positive blood cultures, with blood culture being an 'artificially boosted' clinical specimen, relies solely on the correlation between the targeted genes and the local epidemiological setting. For example, the FilmArray® blood culture identification panel that targets only the *bla*_{KPC} carbapenemase gene could be of interest in areas where this gene is highly prevalent (Italy, Greece, etc.). Frequently, assays targeting the clinical specimen mix the identification of the causative bacteria and the most relevant associated resistance genes. This tendency could at least decrease the possibility of multiplexing the resistance genes for technical limitations. However, in considering the culture as the reference technique, the performances of those molecular tests are excellent if the resistance gene is included in the test panel, with the sensitivity being 100% [97]. Comparative studies basically showed similar and excellent performances for the different commercial kits [80].

4.1.4.2.2. Clinical specimens with a high diversity of bacteria (stools, rectal swabs). Compared to the culture-based method, the performance of molecular methods, and particularly their respective sensitivities, is still a matter of debate. In fact, the culture-based method is considered the gold standard in terms of sensitivity, especially if combined with a pre-enrichment step. Recently, data that were provided about NGS technologies, particularly shotgun metagenome sequencing, question the validity of culture-based techniques as the gold standard [99, see discussion also later].

The detection limit of culture-based approaches for ESBL-producing or carbapenem-resistant *Enterobacteriaceae* in pure culture when using the best selective plates is approximately 10 CFU/ml [93,100].

Performance characteristics from molecular methods vary significantly according to the type of assay; the limits of detection for clinical specimens vary from 10 CFU/ml to 1,000 CFU/ml [78,93]. Global performance should be adjusted to the bacterial DNA extraction rate, which may vary according to the nature of the strain, the putative inhibitory properties of some specimens (stool, blood, etc.), and the number of resistance genes. Some home-made real-time PCR assays reach

performances as high as those of culture-based methods, with detection limits evaluated at 100 CFU/ml of feces. In some cases, the molecular method is 10- to 100-fold more sensitive than the culture-based strategy [77,93,101,102]. The significance and interpretation of a molecular positive-culture negative screening test still constitutes a challenge to clinical microbiology [77,78,97]. Overall, a home-made PCR targeting a single β -lactamase seems to be more sensitive than multiplexed commercial assays; the competitive and technical requirements for administrative agreement lead to increased values in the limits of detection [93].

Several studies have reported suboptimal sensitivities when dealing with mixed gram-negative blood cultures, or when a single strain carried several resistance genes [80,103]. Beyond the previously reported difficulties in including all the variants of a large family of β -lactamases, for example, in *bla*_{CTX-M}, competition may occur when different targets must be amplified from the same specimen [80]. This pitfall may lead to false-negative results and could have clinical consequences. Obviously, the risk of false detection results for polymicrobial specimens is shared by the different methods (culture, biochemical testing, MALDI-TOF analysis, and molecular screening). Microbiologists should modulate their confidence in the results according to the nature of the specimen, whether it is a pure subcultured isolate, primoculture, or noncultured clinical specimen. Some recent assays seem to solve this issue, such as the Luminex® assay [94].

4.1.4.3. Specificity

4.1.4.3.1. Specificity for pure culture. Once the panel is sufficiently large and adapted to local epidemiology, the real remaining challenge for molecular methods is the specificity, or the capacity of an assay to separate the natural chromosomally or plasmid-encoded narrow-spectrum β -lactamases from the extended-spectrum, acquired ones. This issue is well established for cephalosporinase coding genes such as *bla*_{AmpC}, *bla*_{ACC}, or *bla*_{DHA} and constitutes a challenge for some molecular methods [104]. Moreover, among the variety of β -lactamases identified in *Enterobacteriaceae*, a high number of variants from the same class have sometimes been described; for example, more than 300 members of OXA enzymes have been reported. In some cases, and as noted earlier for OXA-48 variants, the acquisition of a single or few mutations may instead lead to an expanded or narrowed hydrolytic activity [87]. This phenomenon is also known in ESBLs that are derived from their narrow spectrum counterparts in the TEM and SHV families. This issue definitely constitutes a real challenge for the design of specific primers and probes [80]. For example, the Check-ESBL assay (Check-Points Health) correctly differentiates among the narrow-spectrum *bla*_{TEM} and *bla*_{SHV}-encoding genes from the variants that encode ESBLs. However, the same technology fails to distinguish some acquired and plasmid-borne *bla*_{AmpC} genes from their intrinsic and chromosomally encoded counterparts. This challenge was clearly addressed by the most recent assays [79,94]. For some assays, the subtyping of the β -lactamase is available, as it is for the *bla*_{CTX-M} families [94]. Unfortunately, the detection of overexpressed natural enzymes through a

mutation in the regulator genes, as is the case for *bla*_{AmpC}, could not be detected without a sequencing approach.

4.1.4.3.2. Specificity for clinical specimens. The vast majority of the PCR methods identify their targets (the genes) without identifying the surrounding genetic environment. This drawback was previously reported with MRSA and the confounding coinfection or co-colonization with methicillin-resistant coagulase-negative *Staphylococci* and methicillin-susceptible *S. aureus*. Additional targeted genes have been shown to overwhelm these pitfalls, for example, in the Xpert® MRSA/SA BC or SSTI that included an additional target corresponding to the junction between the staphylococcal cassette chromosome *mec* gene (*SSCmec*) and the part of the *S. aureus* genome where the *SSCmec* is supposed to be inserted, namely the *SSCmec-orfX* junction. Furthermore, the detection of a gene without phenotypic correspondence constitutes a well-known pitfall of DNA-based strategies [81]. Combined with low prevalence for the targets, the positive predictive value of the molecular test may dramatically decrease to as low as 5–10% [17]. This drawback is particularly relevant when rich microbiota, such as that of the digestive tract, is screened for CPE. As previously described for the immunological methods, this type of pitfall has been identified recently for the detection of β -lactamase genes as follows: nonpathogenic *Shewanella* species harboring a *bla*_{OXA-48}-like gene (its natural progenitor) led to a false-positive molecular result in a patient with a history of *bla*_{OXA-48} *K. pneumoniae* [73]. Moreover, the increasing prevalence of nonfermentative gram-negative bacteria harboring carbapenemase genes such as *bla*_{VIM}-positive *P. aeruginosa* is a source of confounding results when a screening program is implemented for infection control purposes [105]. Thus, in a large screening cohort of 3644 patients and 16,296 samples, the number of positive CPE patients was low ($n = 43$), and 4 patients carried a VIM-positive *P. aeruginosa*. Ten percent (4/43) of patients were initially and falsely identified as CPE-positive, leading to confounding isolating measures and messages toward patients, their families, and health-care workers [106]. Moreover, the positive predictive value of the screening was only 86% [106]. A lower positive predictive value of 50% for the screening was reported by Simnet et al., with PCR-positive culture-negative cases exhibiting mostly high threshold cycle values ($C_T > 39$) [107]. The authors suspected low-level colonization below the limit of detection for the culture-based screening method or nonspecific signal. This finding contrasts with *in vitro* studies that support a higher sensitivity in bacterial culture. Regarding the clinical specimen that required a semiquantitative interpretation of the cultures, for instance, the respiratory tract specimens, the cut-off loads (10^3 for a protected distal specimen, 10^4 for a bronchoalveolar specimen, and 10^6 for sputum) are significantly higher than the limits of detection for the molecular method. This difference leads to confusing discrepancies [104]. Nevertheless, the follow-up of these PCR-positive-culture-negative patients is questionable (see later).

4.2. The RNA-targeted molecular approach

One of the primary disadvantages of the DNA-targeted approach corresponds to a lack of differentiation between

silent and expressed genes or between dead and living bacteria [81]. The replacement of DNA by RNA targets overcomes this problem and diminishes the putative gaps between genetic and phenotypic results. One manufacturer has developed a kit for the detection of *bla*_{KPC} variants (NucliSENS EasyQKPC test®, bioMérieux), and its TAT is 2 h [108].

4.3. The PCR electrospray ionization mass spectrometry (PCR/ESI MS) approach

This technology is based on the ability of some very powerful mass spectrometry instruments to accurately measure the exact molecular masses of small PCR products at less than 500 bp [109]. After that, advanced software reconstructs the sequence of the DNA fragments, allowing for their accurate identification. Given the example of the former Septifast® assay (Roche Diagnostics, Basel, Switzerland), these approaches do not require a culture step, and they are time- and cost-effective. A fully automated system is also available (PLEX-ID; Abbott Bioscience), with the corresponding first-published data about bacterial identifications being quite encouraging, and the TAT from clinical samples being 4–6 h. Preliminary results for resistance gene analysis support the use of this technology to identify the *bla*_{KPC} genes [110]. Nevertheless, the cost of the system (ca. 200,000 USD) remains prohibitively high for a large majority of clinical laboratories.

4.4. The clinical impact of the molecular approach

4.4.1. The individual impact

Beyond theoretical and intermediary results such as the TAT, the cost savings, and the delayed optimization of antimicrobial therapy, the impact of molecular methods on the clinical outcome is poorly established. A recent review and meta-analysis showed that (i) there was a relatively low number of studies pertaining to clinical outcomes, (ii) the one combined cohort showing a significant reduction in mortality when rapid molecular testing is associated with direct communication, and (iii) there was a lack of significant impact on mortality for rapid phenotypic techniques associated with direct communication [6]. More specifically, the molecular assay was a peptide nucleic acid fluorescent *in situ* hybridization that was performed directly on a positive blood culture in comparison with a Gram stain, and the phenotypic approaches that failed to reduce the mortality consisted of rapid antimicrobial susceptibility testing with Vitek® (bioMérieux, Marcy l'Etoile, France) or Microscan® (Beckman Coulter, Sacramento, CA, USA).

4.4.2. The collective impact

Although it seems like common sense, the impact of decreased antibiotic pressure in response to a rapid molecular testing strategy was not established. Moreover, the impact of this approach in terms of infection control, i.e. the decrease of the occurrence or the duration of outbreaks has not been concluded; an intermediate positive impact such as the time until contact isolation has nonetheless been reported [111].

4.5. Advantages of the molecular-based approach

Compared to biochemical and immunologic approaches, the primary advantage of the molecular-based approach is its capacity to be used directly from complex clinical specimens including rectal swabs and stools. The current trend is the development of multiplexed real-time PCR that allows for the detection of different relevant targeted genes leading to the identification of the causative agents and the primary frequently acquired resistance genes. This approach rapidly provides critical data about natural and acquired resistance traits. This information is of paramount importance for clinical decision-making, especially for life-threatening infections. The total hands-on time from the arrival of the specimen to the test result actually varies, but it does not exceed 1 h, as opposed to a median time of 2 or 3 days when using conventional methodologies requiring the culture of the specimen on a selective medium followed by antimicrobial susceptibility testing. The ability of some of these tests to be implemented as POCT will surely improve their availability and TAT. If they are available in clinics, those tests can offer an accurate diagnosis beyond laboratory open hours and/or decrease the turnaround time in limiting the transport delay from the clinical ward to the laboratory.

The low level of resistance conferred by some genes sometimes leads to a lack of phenotypic detection. However, a clinically significant level of resistance may appear under antibiotic selective pressure, which has been reported for VRE [112]. In this particular case, the molecular detection of the genes clearly provides an advantage. When considering β -lactamase detection, some enzymes do confer a low level of resistance. As an example, some OXA-48-producing enterobacterial isolates do exhibit a low level of carbapenem resistance that could escape phenotypic detection. In fact, the MICs of carbapenems from some OXA-48-like producers may be ca. 0.25–0.5 mg/l, which are below the cut-off limits for determining carbapenem resistance. Due to the high transmission rate of the epidemic plasmid bearing the *bla*_{OXA-48} gene, the level of resistance could be significantly increased if the plasmid is transferred in another strain possessing another background that might exacerbate the resistance trait, giving rise to much higher MICs. Regarding infection control, molecular tests can be performed directly in complex microbial communities, including stools or rectal swabs. If combined with bacterial identification, the molecular panels constitute an efficient test to help clinicians to rapidly implement the most appropriate antimicrobial strategy. As a surrogate method for DNA sequencing, the PCR/ESI MS method distinguishes among single nuclear polymorphisms and allows for the fine subtyping of the resistance gene.

Notably, the fact that molecular methods may either detect viable or nonviable microorganisms may be considered as either an advantage (higher sensitivity) or a disadvantage (for having a lack of significant clinical relevance).

4.6. Drawbacks of the molecular approaches

4.6.1. The limited number of targeted genes; walking in a moving landscape

As previously reported, the evolving diversity of β -lactamase genes constitutes a challenge when addressing specific

methods such as gene-targeted PCR. The multiplexing capacity of commercial kits is limited. The choice of the gene panel depends on which ones are considered or defined to be the most relevant in a given geographical area and within a given period of time [111]. Numerous commercial kits were developed for the U.S. market, with focus on the *bla*_{KPC} gene, which is the most prevalent carbapenemase gene in this area; unfortunately, assays such as the FilmArray Bloodculture Identification® panel have stirred little interest in the geographical area where other carbapenemase genes are more prevalent. Likewise, the first Cepheid panel, namely the Xpert MDRO assay®, did not include the *bla*_{OXA-48} that was not prevalent in the U.S. [113]. Recently, this gene has been included in the cartridge and the kit that was renamed Xpert Carba-R®. However, failures to detect producers of OXA-48 variants, such as OXA-181 [75,114,115], have been reported. An updated version of the test had to be developed that encompasses the *bla*_{OXA-181} and *bla*_{OXA-232} variants [76]. The molecular approaches are challenged with OXA-48 enzymes since the corresponding genes encompass many variants [87]. One consequent problematic issue may be the nonrecognition of some variants on one hand and the recognition of variants that exhibit different hydrolytic properties on the other hand. In recognizing the hydrolytic variants, molecular assays do not differentiate between a *bla*_{OXA-48} gene encoding a β -lactamase-hydrolyzing carbapenem, but they do spare expanded-spectrum cephalosporins and a *bla*_{OXA-163} gene encoding a β -lactamase sparing carbapenems; conversely, they hydrolyze expanded-spectrum cephalosporins. As previously described, some microarray assays combine a very large panel of targeted genes; nevertheless the cost, dedicated materials, and TAT could limit their use [79]. Some technical solutions combine the PCR amplification of a large number of targets followed by a new generation of sequencing (see Section 4.4.1).

Beyond the diversity of genes, the inability to detect a new (possibly emerging) gene constitutes a major limitation in the molecular approach. As an example, the recently identified class A carbapenemase FRI-1 that was recovered from an *Enterobacter cloacae* isolate is not recognized by any available molecular kit on the market [116]. This finding has also been demonstrated for other bacterial species such as in *Staphylococcus aureus* with the under-recognition of the emerging *mecC* gene among methicillin-resistant *S. aureus* [87]. The changing epidemiology of the β -lactamase-encoding genes will surely remain a challenge for industry and for clinical laboratories. To complicate this issue further, the time required to modify a commercial test usually exceeds 1 year. Thus, one of the remaining issues is the predictive negative value of a negative test. If the targeted gene was previously identified (e.g. for a contact patient) and was included in the panel, this value is high and leads to a univocal decision. Conversely, a negative result for the first screening of an unknown patient or the typing of an unanticipated resistant strain should be interpreted according to the adequacy between the epidemiology in the resistance supports of patient origin and the panel of the assay. Obviously in this case, especially when the patient comes from a distant country in which the epidemiology of resistance genes is poorly understood, the interpretation of a negative result depends of

the clinical severity (if it guides the choice of an empirical therapy) or the care volume and risk of transmission (if it guides the choice of the infection control measures).

4.6.2. The reasons and consequences for discordant/false results

4.6.2.1. Discordant results. Discordant results raise a question about the gold standard technique that should be considered during pathogen detection. We have reported some factors that are associated with lower sensitivity in the molecular methods. The culture-negative molecular testing-positive-discordant cases could range from 5% to 55% [77]. As a result of the increasing sensitivity of the most recent molecular assays, including the **next genome sequencing (NGS)** approach, the culture-based method could miss antibiotic-resistant bacteria if they had a low inoculum. For example, in Lau et al., the molecular method was 10-fold more sensitive than the culture-based method for two *K. pneumoniae* strains carrying *bla_{OXA-48}* and *bla_{OXA-244}* genes [77].

Beyond the possible lack of sensitivity of the culture methods, several other explanations could be proposed as follows:

- the presence of additional nontargeted bacteria in the specimen (non-enterobacterial gram-negative bacteria such as *P. aeruginosa* or *Shewanella* sp.) that carried the targeted resistance gene, as found, for instance, for *bla_{OXA-48}*, *bla_{VIM}* or *bla_{KPC}*, and that are not detected by culturing;
- the presence of dead or noncultivable bacteria: one of the five discordant patients in the study by Antonelli et al. had a history of KPC-producing *K. pneumoniae* colonization [78];
- the presence of bacteria that harbor but do not express the targeted resistant gene, as in the intrinsic *bla_{OXA-51}* from *A. baumannii* that is responsible for carbapenem resistance, only if a strong promoter is provided by the insertion of the insertion sequence *ISAba1*. This issue may be solved by focusing on RNA rather than DNA detection; and
- the presence of bacteria that harbor the targeted resistant gene but express it at a very low level, and the use of selective culture plates with predefined antibiotic concentrations could lead to the false-negative screening of a culture.

4.6.2.2. Clinical consequences of discordant results. For clinical care, the very serious error of a false-negative result could lead to inappropriate therapy and a putatively pejorative outcome. Nevertheless, if the false-negative result is due to a nonexpressing gene or a very low level of phenotypic resistance, then the clinical consequences should be limited. For instance, the presence of a gene that codes for a carbapenemase may not exclude the carbapenem from the therapeutic choice. In fact, patients infected by a CPE (VIM producer) for which the meropenem MIC was ≤ 4 mg/l might benefit from carbapenem therapy, at least in combination [117]. Beyond carbapenem therapy, the detection of a carbapenemase gene could nonetheless encourage clinicians to prescribe a combination therapy. However, a false-positive

test (i.e., a major error) could lead to an unnecessarily broad spectrum empirical therapy that would then lead to the selection of resistant strains.

4.6.3. The impact of the culture-independent diagnostic assays on the survey study

For other public health domains, the development of culture-independent diagnostic tests raises questions about its impact on surveillance studies [118]. We believe that culture-based methods remain necessary for the following reasons: (i) the need to compare the strains that are responsible for outbreaks and (ii) the lack of absolute parallelism between the presence of a gene and the occurrence of resistance. Clinical isolates usually express combined mechanisms of resistance that will be difficult to show by using molecular techniques, including whole genome sequencing. One of the good examples corresponds to the overexpression of efflux proteins and decreased outer membrane permeability in the *P. aeruginosa* species that may lead to multidrug resistance but cannot be identified by molecular methods.

4.6.4. The cost

Molecular methods remain more expensive than biochemical tests (from 30 to 60 USD vs. 5 to 10 USD). Nevertheless, this cost must be balanced against the TAT and the technical time (Table 4). This cost increases when invalid/inhibited samples were identified. The percentage of unresolved samples that required retesting after, for example, a freeze-thaw cycle, is scarcely reported, but it could reach 3.2% of the total sample number [78]. Sometimes, a second testing step is still insufficient for obtaining an informative result. In Lau et al., 52% of the invalid samples could not be resolved even after additional testing [77]. Pooling the test specimens to minimize the overall cost significantly decreases the detection rate if the inoculum of the targeted gene is originally low in one of the specimens (e.g. 150 CFU per swab) [77]. The development of home-made techniques may result in

Table 4. Comparative advantages and disadvantages of molecular versus biochemical rapid tests from the individual and public health perspective.

	Molecular tests	Biochemical tests
Applicable directly on clinical specimen	Yes	Yes (urines and blood)
Necessity of viable organisms	No	Yes
Broad range screening	No	Yes
Nonspecific or nonconclusive result	No	Yes
Discover of new resistance mechanism	No	Yes
Technically skill requirement	+*	No
'Hands-on' technical time	Variable	Low
Turnaround time	1 to 2 h	From 2 min if positive to 2.5 h if negative
Cost per test	23–150 USD	2–10 USD
Detection of low-level resistance	Yes	Yes
Detection of cryptic resistance gene	Yes	No
Special equipment requirement	Yes	No
Additional testing required for definitive identification	No	Yes

*Except Cepheid.

cheaper costs, but those techniques may not correspond to modern requirements.

5. Future approaches

5.1. The NGS approach: determining the 'resistome'

5.1.1. Principles of the NGS approach

Next to the PCR revolution in the early 1990s, NGS technology is changing the field of microbiology. The NGS technology is a high-throughput sequencing method that allows for the concomitant determination of the nucleic acid contents of thousands or millions of genes [109]. It can determine the complete genome of a cultivated bacterium in only a few days instead of several months. The contribution of this 'Whole Genome Sequencing (WGS)' approach was demonstrated during an outbreak of enterohemorrhagic *E. coli* in Germany in 2011. NGS technologies aid in understanding pathogenesis and identifying resistance genes, developing diagnostic tools, and improving patient care [119]. Interestingly, this strain carried a *bla*_{CTX-M-15} ESBL that was characterized because of the WGS data analysis. Next to the WGS approach, the NGS technologies help researchers to explore the microbiome; the metagenomic analysis of a specimen consisted of sequencing its entire DNA content from all the cultivated and uncultivated bacteria. This analysis could be focused on 16S rRNA gene sequencing by using a PCR approach ('16S rRNA metagenome sequencing' or 'metagenomics'), allowing an identification at the genus and sometimes species levels of the different bacteria present in a specimen. More interestingly, the 'shotgun metagenome sequencing' approach allows for the sequencing of the whole DNA extract from an uncultivated specimen, and it is not limited to 16S rRNA gene sequencing. Beyond a more accurate bacterial identification, this approach enables investigators to highlight the presence of resistance or virulence genes without the need for a culturing step. Nevertheless, the difficulties associated with the manipulation and interpretation of important amounts of data have now replaced the technical issues (see *later*). Finally, the sequencing of the whole mRNA pool of a strain or a specimen helps in capturing the image of all the genes that are expressed at a given time ('the transcriptomics') [99].

The technical specifications for the different methods used for WGS were out of the scope of this review article and have been discussed before [109,120]. In brief, since the end of 454 technology (Roche Diagnostics, Basel, Switzerland), several instruments have been made available, with each of them having their pros and cons. Next to Illumina technology (San Diego, CA, U.S.A.), the Ion Torrent semiconductor sequencing (ThermoFisher Scientific, MA, U.S.A.) and Pacific Biosciences (CA, U.S.A.) platforms bring alternative solutions, with the Pacific Biosciences product generating very long reads that facilitate *de novo* assembly (see *later*) [109,120]. To constitute a real revolution that can be implemented within clinical microbiology, a new technology has to be available for the majority of scientists, including clinical microbiologists. The following two primary features of the NGS revolution reach this goal: (i) the miniaturization of the instruments, with the marketing of

bench machines such as the previously produced 454 Junior and the MiSeq and Ion Torrent instruments, and the still-under-assessment ultra-miniaturized MinION Mk1 (Oxford Nanopore, Oxford, U.K.) and (ii) the decreasing cost of the process allows for the sequence of a whole genome for less than 150 euros [119]. In parallel, the availability of commercial software in the pipeline to sort out and classify the huge amount of sequencing data and user-friendly websites to identify genes of interest will definitively place WGS at the center of clinical laboratories in the near future.

Regarding antimicrobial resistance, the NGS technologies could improve the detection and characterization of the mechanisms and the genetic support and background in terms of accuracy and rapidity [119]. Beyond pyrosequencing, which has been shown to be useful in differentiating the variants of a core resistance gene [121,122], the NGS approach allows for the determination of the whole genome of a bacterium that could be subsequently challenged against a database that contains a list of resistance genes [119]. It was initially developed for culture-pure isolates, and this technology was then extended to clinical specimens that contain only one bacterial species in sufficient quantity (urine) and then more complex specimens such as stools or respiratory tract specimens. The use of the NGS in such complex environments highlights the critical issue of (i) DNA extraction from different bacterial populations and (ii) the technical and *in silico* cleaning of human DNA. The NGS approach leads to the identification of the resistome from a strain or a microbiome, that is, the pool of genes implicated in the natural and acquired antimicrobial resistance in bacteria.

5.1.2. The resistome, a surrogate for the phenotypic and molecular characterization of resistance?

Accessing the resistome of bacteria allows researchers to answer the questions in this review in a theoretical sense. Several publications reported high consistency between WGS data and antimicrobial susceptibility testing results among *Enterobacteriaceae*, especially *E. coli*, *K. pneumoniae*, and *Salmonella sp* [123–126]. The correlation between the WGS-based resistant gene armamentarium of a bacterium and its phenotypic antibiotic susceptibility appears to be as high as 99%, reaching the error rate targets of the U.S. FDA for marketing approval (<1.5% very major discrepancy rate and <3% major discrepancy rate). User-friendly bioinformatics approaches such as the ResFinder® website from the Center for Genomic Epidemiology are now available, with some for free and some other requiring an additional payment for use [99,102,127]. After the production of raw data by the NGS instruments ('reads'), bioinformatics processing could be performed directly or after being assembled into consensus sequences ('contigs'). A trimming step has to be performed *in silico* to clear the biased reads from the data set (see *later*). In focusing on β -lactam resistance, the following challenges should be addressed: (i) the detection of acquired genes, (ii) among them the separation of narrow- and broad-spectrum β -lactamases that differ by point mutations, and (iii) the detection of mutations/deletions in natural genes and/or in promoter regions that could lead to acquired resistance. Obviously, this last class of genetic events is difficult to detect, requiring

high accuracy for single-nucleotide polymorphism ('SNP') identification and large contigs to obtain the upstream sequence of a putative resistance gene.

To address the putative clinical implications of an exclusive *in silico* approach, a new committee from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) was created in 2015, which was called the 'EUCAST subcommittee of the role of WGS in antimicrobial susceptibility testing of bacteria' (<http://www.eucast.org/organization/subcommittees/>). The first reports from this committee are pending.

Moreover, the NGS approach has been shown to be effective for detecting MDR bacteria from a whole microbiome. In light of the most recent data collected by shotgun metagenome sequencing, the assignment of the gold standard label to culture-based methods could be questioned. In the work of Andersen et al., half the inpatients experienced colonization with an MDR bacterium that was not identified by culture but was highlighted by fecal metagenome sequencing [99].

5.1.3. Advantages of the NGS approach

The NGS approach displayed several advantages compared to the culture methods or targeted molecular assay. First, the NGS may characterize pathogens that cannot be cultured because of inappropriate growth conditions or previous antimicrobial treatment. Moreover, unlike the targeted molecular assay, the NGS approach could detect mutations or resistance genes at extremely low levels in a bacterial population. This capacity leads to the designation of the 'ultra-deep sequencing' concept that allows researchers to identify the presence of mutants at a level of less than 1% of the population. Nevertheless, this approach requires high coverage (i.e. a number of sequencing products) of the relevant zone of the genome (more than 10,000-fold) through an initial specific PCR-based amplification step. This approach was successfully implemented in the virology domain to detect minor populations of mutants among HIV or HBV/HCV populations. The presence of these mutants could predict the clinical success of an antiviral regimen. This approach could be implemented to target the hot spots of mutations among bacterial genomes, detecting some genetic backgrounds that could lead to the emergence of resistant subpopulations in some specific clinical settings (high inoculum, presence of exogenous material, immunosuppressed status of the patient, long-term antimicrobial therapy, etc.). In comparison to targeted molecular methods, the WGS could also detect some new β -lactamases or new mutations in known genes as reported in the work of Kos and colleagues, who were interested in the resistome of *P. aeruginosa* [128]. Beyond the acquisition of new genes, the putative difficulty in detecting the overexpression of chromosomal β -lactamase as AmpC could be overcome. For example, in a study by Tyson et al., the ceftoxitin resistance of three *E. coli* strains that did not show any other resistance mechanisms was explained by the detection of a mutation in the *ampC* promoter [125].

Beyond the resistome, the WGS of a given strain allows access to its whole genetic heritage, including virulence gene identification, phylogenetic tree building, the localization of mobile genetic elements that may support the resistance

genes such as plasmids, phages and integrons, genetic background characterization (such as MLST typing), and outbreak investigation using SNP quantification [125,128]. These additional data are available without extra charges, requiring only the *in silico* analysis of the genome. Sometimes, all these possibilities are available at the same website (e.g. the Center for Genomic Epidemiology, <http://www.genomicepidemiology.org/>).

5.2. Pitfalls of the WGS approach

As previously reported, the availability and cost of WGS technology should not be a hurdle in the near future. The delay required for data production, which was a limitation for clinical purposes, decreases in parallel to the decrease in cost and the increase in indications. At present, the sequencing and bioinformatics process could be performed with a three-day delay that is competitive with the culture approach, but not with targeted molecular methods that do not require additional sequencing. Some technical limitations of each type of NGS instrumentation have been clearly identified. For example, the pyrosequencing method used in the 454 instrumentation and the semiconductor sequencing used by the Ion Torrent system fail to generate reliable sequences of homopolymers higher than 4 or 6 nucleotides in length, respectively. These drawbacks could be managed by combining different technologies to confirm or correct the sequencing of some particular regions of the genome. Nevertheless, this solution increases the final cost of sequencing.

To date, the primary limitation of the NGS approach has clearly been the bottleneck that occurs during the interpretation of this huge quantity of data for gene identification and phenotypic transcription. If single gene characterization can be performed easily using available databases, then the interpretation of the results has been previously reported to be performed with caution [128]. Some drawbacks still persist such as the quality of the reference database or the parameters used to produce and compare sequences. The results of the *in silico* analysis could differ according to the software and database [126,129]. For microbiologists who are not skilled in bioinformatics technologies, the different software types that are provided by instrument manufacturers and web-based platforms constitute some type of 'black box'. For example, the *de novo* assembly step that leads to the building of contigs without a reference strain could lead to the loss of information; in fact, if a resistance gene is spread over two contigs, it may not be detected even though the coverage cut-off is lowered (60% for the ResFinder software) [126]. The use of reads without an assembling step should be of interest [126]. For clinical applications, the lack of control over this part of the analysis by biologists clearly constitutes a drawback. Notably, the databases should be regularly updated to include new resistance genes or mutations [129]. If a database is free of charge for users, its durability might be compromised if financial support fails. Obviously, the majority of the well-designed studies that are published have relied at least in part on 'home-made pipelines'; these approaches require bioinformatics skill and cannot be translated in clinical laboratories. As times change, some scripts using the Perl or Python languages

are now available as supplementary data in nonspecialized journals. Some commercial solutions are under development to include the NGS technology in commercial *in vitro* diagnostic CE-marked systems to improve the performance of the targeted PCR approach, such as the Pathogenica HAI BioDetection system (Pathogenica Inc., Boston, MA, U.S.A.) [130]. Regarding the metagenomic shotgun approach that could provide the Holy Grail in terms of detecting resistance genes from clinical specimens, the technology currently presents some drawbacks as follows: a huge amount of data to produce, analyze, and save; the need for bioinformatics and statistical skills; and so far a statistical association between resistance genes and bacterial species that leads to 'MDR potential' rather than the definitive characterization of the whole genome of an MDR bacterium [99]. However, in light of the complexity of the MDR carriage and particularly the within-host diversity, the culture method could be considered as an excessively simple solution for a complex issue [131].

As previously reported, the detection of resistance traits based on mutations in promoters or the coding regions of natural genes are more difficult to detect than exogenous genes. Mutational events linked to *bla*_{AmpC} overexpression have been properly identified in several published works. However, β -lactam resistance due to mutations in porins, as in imipenem-resistant *P. aeruginosa*, or insertion sequences that provide a strong promoter, as in *Bacteroides* sp., should be improved, requiring high resolution and/or complete genome sequencing [132]. In a study by Kos et al., the genetic support of the meropenem resistance was not identified in 15/154 *P. aeruginosa*; additionally, the quality of the OprD sequencing was insufficient for eight isolates to predict the meropenem susceptibility *in silico* [128]. The insufficient quality of the sequencing, i.e. the high number of contigs or the low coverage of some genomic regions, could limit the performance of the method. Thus, the detection of the genetic support such as the integrons or plasmids could be incomplete or inconclusive. Some software that allows for the detection of plasmids from raw sequencing data is available, such as Plasmifinder. This free tool detects replicons using a curated database, identifying the part of the genome that could be part of a plasmid [133]. The website also subtypes the identified plasmids according to a plasmid MLST analysis. Nevertheless, the comprehensive characterization of the larger plasmids still requires the extraction, separation, and individual sequencing of each plasmid unless the selected technology provides very long reads, such as the use of PacBio® instrumentation.

Moreover, some complex supports of resistance that involve cooperation between several genes and associated regulators are probably more difficult to identify than mechanisms based on the presence/absence of a resistance gene. For instance, the results of the resistome approach could vary according to the nature of the given mechanisms. Kos et al. sequenced 390 strains of *P. aeruginosa* from various parts of the world, and they found that the consistency between their culture-based susceptibility data and the resistome was lower for aminoglycoside resistance, implying more gene expression variations than for β -lactam resistance, such as for meropenem resistance (60% vs. 91%, respectively) [128].

A large number of these pitfalls could be fixed by improving the quality of the sequencing process. To date, the best solutions have combined technologies that provide very long reads such as the PacBio® instrumentation to build the scaffold of the genome and the putative plasmids, and high coverage methods such as the Illumina® approach. Nevertheless, this combination increases the cost and the time needed to interpret the data. Supplementary technical approaches such as transcriptome analysis using RNA-seq or microarray gene expression could overcome the issues associated with gene expression and regulation, especially for some bacterial species. Antimicrobial compound combinations such as *P. aeruginosa*/meropenem provided insufficient correlations between the presence of a gene and its phenotypic resistance. In fact, this transcriptome profiling provides some additional interesting data such as a correlation between the expression of *oprD* and meropenem resistance [134]. However, the correlation between the *oprD* mRNA level and protein production was only partial, and it was putatively linked to post-transcriptional regulation. This domain falls within the research realm and is it not relevant to clinical settings at present.

5.3. Other possible technologies

In the near future, some emerging technologies could provide new solutions for detecting β -lactam-resistant bacteria [135]. For example, regarding the phenotypic approach, flow cytometry or microfluidic assays are promising new technologies that are benefiting from advances in the detection of antimicrobial activity at the individual bacterial level [136,137]. Other technical progress could improve the performance of current methods; for instance, the BYG Carba Test® is a new electrochemical assay that reacts to the pH modifications associated with imipenem hydrolysis by carbapenemases, and it might fit with the Carba NP test principle that is based on a pH indicator color change [138]. Numerous other molecular and non-molecular tests have arisen; thorough and multiple comparative studies against reference strains and methods must confirm their putative interest and clinical relevance.

6. Expert commentary

The detection of β -lactam resistance from a clinical specimen or a bacterial colony is a top priority for clinical microbiologists, since β -lactam antibiotics remain the most prescribed antibiotics for humans. A large panel of technical solutions is increasingly available. Depending on the context, the objective may be the detection of an emerging and transmissible resistance trait or the evaluation of the clinical success probability of an antimicrobial therapy. This dual objective represents the individual and collective approaches to the problem. This individual purpose primarily relies on the evaluation of the intrinsic susceptibility of the bacteria for the agent, which is reflected through the MIC value. To date, there has been no other parameter that could compete with the MIC determination. Due to the unavoidable amount of time required to obtain the MIC

values of classical antibiotic susceptibility testing, the detection of surrogate targets as other phenotypic traits (biochemical activity or protein detection) or genetic characteristics constitute an acceptable alternative, and thus they join the collective objective.

The choice of a given test should be based on the performance, reliability, and cost of the given detection assay (Table 4). Considering the screening approach from an isolated colony, no molecular-based methods could be considered as a blind first-line test. The future development of the NGS solution could modify this statement, although the delay, cost, and interpretation criteria need to be significantly improved. This position is closely related to the never-ending diversity of β -lactamases. Therefore, the choice of biochemical test should rely on its intrinsic performance, which could be challenged in numerous comparative studies from high-level publications. To date, the Rapidec Carba NP test® and the ESBL NP Test® that were challenged in more than 60 publications constitute an undisputed reference in terms of their performance/cost ratio. Due to its wide implementation in clinical laboratories, the MALDI-TOF MS approach is also an interesting alternative strategy, even though the reference in terms of a protocol is not established at the moment. As a second line of investigation, when the β -lactamase type must be identified or when putative contact carriers have to be screened after the identification and characterization of an index case, the narrow-targeted molecular or immunologic assays constitute acceptable (however imperfect) solutions. Regarding the performance of these different strategies when clinical specimens are considered, the molecular approach could significantly improve the turnaround time of multidrug-resistant organism detection from stools; nonetheless, these results must be controlled using a culture-based strategy. For the clinical specimens that exhibit a high burden for a unique pathogen (e.g. a positive blood culture or urine sample), the performance of rapid phenotypic testing should be confirmed by additional studies, but they could challenge molecular assays.

This hierarchy could be debatable and questioned according to the availability of new approaches, allowing a faster determination of the MIC values or an exhaustive molecular screening.

Considering the costs of some of these assays, which may be substantial, economic studies focusing on the clinical relevance of these supplementary expenses must be implemented. Clearly, it will be difficult to demonstrate any clear and significant impact from these assays, as in cases for undisputed and new relevant technologies such as MALDI-TOF. Beyond the technical consideration and performance of the test, the previously published works have supported the paramount importance of their incorporation into a virtuous circle, including the release of local guidelines, and the substantial counseling availability of infectious disease specialists.

7. Five-year view

The future of antibiotic efficacy is threatened by the global spread of MDROs. Taken together, antimicrobial misuse, cross

transmission, and environmental contamination will lead to an uncontrollable and irreversible situation, particularly in developing countries. The continuous increase in international exchange promotes the spread of MDRO. From the pharmaceutical industry perspective, the pipeline of new antimicrobial products is unlikely to compensate for the increasing need for effective antibiotics. There is only one option that actually remains and that is saving antibiotics for triggering and adapting them as soon as possible for antimicrobial therapy, in combination with a strict infection control policy. For these purposes, all the field stakeholders, i.e. infectious diseases specialists, infection control practitioners, pharmacists, and clinical microbiologists, shall rapidly establish decision algorithms for mixing individual patient risk-factor analysis and different technologies as culture-based, biochemical, and molecular methods. These algorithms would be adapted to the local epidemiology, patient recruitment, and technical and financial local capacities. Moreover, the development of antimicrobial stewardship and infection control teams will continue through the implementation of transversal units of specialists. Pharmacists will pursue a policy of restrictive access to last-resort compounds. The cornerstone position of the clinical laboratory will be strengthened if it has adapted its technical display and daily operation to local needs. Due to the decreasing number of laboratories in favor of large concentrated platforms, we believe in the development of point-of-care technologies that will be available at the bedside. In parallel, the contribution of the cheap and easy-to-use biochemical tests for the characterization of isolated colonies or pure clinical specimens will be strengthened in practical algorithms of antibiotic initial prescription/de-escalation and infection control decisions around the world. The culture-based strategy, which is and will likely continue to be the gold standard reference method for the next 5 years, is going to be increasingly challenged by molecular methods, especially NGS. As exemplified by the latest developments in virology (HIV, HCV, etc.), we believe that the use of companion diagnostics will help to guide clinicians to faster and more adequate antibiotic therapies. To conclude, we believe in a near future of complementary approaches to bacteriological diagnostics, including culture, biochemistry, biophysical, and molecular techniques, depending on the type of bacteria and resistance traits to be identified.

Key issues

- The rapid detection of β -lactam resistance is a key element of antimicrobial stewardship and infection control strategies
- The extreme variability in the genes and variants responsible for β -lactam resistance makes it impossible to limit diagnostics to PCR-based approaches alone
- The biochemical approach is the reference method for first-line screening purposes
- Efficiency and reliability from cultures to clinical specimens are essential
- Upon being integrated into an evidence-based algorithm, their cost is more than acceptable

- Immunological and molecular assays are of interest for identifying the primary resistance genes in case of positive biochemical tests
- During outbreaks, immunological and molecular methods are of special interest to screen patients when the responsible gene (or the corresponding bacteria) is already identified
- In the future, the whole genome sequencing approach will take an increasingly greater place in the detection of resistance genes from strains, clinical specimens or the microbiome
- All bench-based strategies must be included in a bed-based antimicrobial stewardship program

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