1 **The Changing Role of the Clinical Microbiology Laboratory**
2 **in Defining Resistance in Gram-negatives**

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ABSTRACT

The evolution of resistance to antimicrobial agents in Gram-negatives has challenged the role of the Clinical Microbiology Laboratory to implement new methods for their timely detection. The development and spread of multi-drug resistant strains, including those with extended-spectrum β -lactamases (ESBLs) and carbapenemases, have presented major challenges to detection by both conventional and new methods. Methods for more rapid pathogen identification and antimicrobial susceptibility testing, particularly of bloodstream infections, have been developed for use on growing cultures and are in development for use directly on specimens. These methods include fluorescence *in situ* hybridization tests, automated polymerase chain reaction systems, microarrays, mass spectroscopy, next-generation sequencing and use of microfluidics. Review of these methods shows the advances that have been made in rapid detection of resistance in cultures, but limited progress in direct detection from specimens.

Key words: AST, PCR, real time, LAMP, microarray, MALDI-TOF, sequencing, rapid

KEY POINTS

- Antimicrobial resistance in Gram-negatives is a major challenge;
- Multi-drug, including carbapenem-, resistant strains are increasing;
- More rapid methods are needed;
- Considerable advances have been made with rapid genotypic methods;
- Advances have also been made with rapid phenotypic methods.

INTRODUCTION

The role of the Clinical Microbiology Laboratory in defining resistance in Gram-negatives has been challenged by the evolution of resistance to antimicrobial agents as we are no longer able to rely on the efficacy of the empiric use of “broad-spectrum” agents. In particular, development and spread of extended-spectrum β -lactamases (ESBLs; e.g., CTX-Ms) and carbapenemases have presented major challenges. The mutation of narrow-spectrum β -lactamases (which degrade penicillins) into ESBLs (which add cephalosporins and monobactams to their spectrum) has limited the activity of advanced generation cephalosporins. Acquisition of carbapenemases such as KPC, NDM, IMP and VIM results in resistance to virtually all available β -lactams in common use. Moreover, these strains are frequently resistant to many other drug classes, rendering them resistant to typical empiric therapy combinations [1-3].

These challenges have led to the realization of the need for more rapid diagnosis, particularly of bloodstream infections, and for more rapid antimicrobial susceptibility testing (AST). A mean decrease in survival of 7.6% for each hour after onset of infection until effective antibiotics are administered has been reported, as well as a 5-fold increase in mortality when inappropriate antimicrobials were administered within 6 hrs after recognition of septic shock [4]. Recent studies have also documented the value of more rapid diagnosis, which allows earlier appropriate, targeted antimicrobial use [5]. This has been shown to improve patient outcomes, lower mortality, decrease hospital length of stay, lower superinfection and adverse drug reaction rates and decrease costs.

While the rapid detection of bacteria and their resistance mechanisms directly from blood specimens is still an elusive target, this has been achieved on growing blood

cultures, which typically become positive after 18-24 hrs of incubation. Many systems for rapid bacterial identification from growing blood cultures have been developed, such as fluorescence *in situ* hybridization (FISH) tests, mass spectroscopy (MS) and automated polymerase chain reaction (PCR) systems [6]. Many of these systems can also detect antimicrobial resistance genes. For instance, a recent study of an automated molecular system documented the value of one such system, the Verigene Gram-negative blood culture nucleic acid test (BC-GN; Nanosphere), a multiplex, automated test for the identification of eight Gram-negative organisms and 6 resistance markers from blood cultures with a turnaround time (TAT) of approximately 2 hrs. The test correctly identified 95.6% of isolates and detected CTX-M and OXA resistance determinants, with an intervention group having a significantly shorter duration to both effective (3.3 vs. 7.0 hrs; $P < 0.01$) and optimal (23.5 vs. 41.8 hrs; $P < 0.01$) antibiotic therapy [7].

AVAILABLE METHODS

Standard antimicrobial susceptibility test (AST) methods

Conventional AST procedures have been in use for many decades and follow methods and interpretations of various organizations such as European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratory Standards Institute (CLSI) [8, 9], as well as regulatory agencies such as US Food and Drug Administration (FDA) and European Agency for the Evaluation of Medicinal Products (EMA). These organizations and agencies have established “reference” AST methods based on minimum inhibitory concentration (MIC) determination by microdilution and agar dilution, with incubation times ranging from 18 to 48 hrs. Disk diffusion methods have also been standardized by these groups.

Many commercial methods for AST are available and are based on using the above reference methods directly, or by methods correlated to reference methods and providing comparable results. Commercial methods using reference microdilution methods include MicroScan WalkAway (Siemens Healthcare Diagnostics) and Sensititre (Trek Diagnostic Systems). Methods providing results comparable to reference testing include gradient diffusion MIC determination (Etest), Vitek (bioMérieux), Phoenix (BD Diagnostic Systems) as well as rapid versions of MicroScan and Sensititre. Several of the methods have faster TAT than reference methods, and many are automated with machine-generated results. Instruments that record and interpret disk diffusion zone are also available (e.g., BIOMIC V3, Giles Scientific; ADAGIO, Bio-Rad; Scan 1200, Interscience; SirSCAN, i2a).

These reference AST methods also include methods for determination of resistance mechanisms, such as the presence of ESBLs in some *Enterobacteriaceae* using cefotaxime and ceftazidime alone and combined with clavulanate, and the presence of carbapenemases in *Enterobacteriaceae* using lowered carbapenem breakpoints and the modified Hodge test [8]. Many commercial AST systems incorporate these methods and many other tests for detection of these resistance mechanisms are commercially available and will be discussed.

Rapid biochemical tests to detect ESBL and carbapenemase producers

The ESBL NDP test is a rapid (from 15 min to 1 h) and cost-effective biochemical test used to detect ESBL producers. It is based on the recognition of cefotaxime hydrolysis that is inhibited by tazobactam. ESBL production is evidenced by a color change (red to yellow) of the pH indicator phenol red due to acid formation resulting from cefotaxime

hydrolysis that is reversed by adding tazobactam. Its sensitivity and specificity for detecting ESBL-producing *Enterobacteriaceae* are 93% and 100%, respectively [10]. The test has been evaluated on blood culture and urine samples, showing excellent sensitivity and specificity (>98% and >99%, respectively) [10-12].

The Carba NP test was specifically designed to detect carbapenemase producers. It detects a change in pH due to the hydrolysis of imipenem in presence of carbapenemase enzymes in less than 2 hrs. Without the need for any particular equipment, β -lactamases are rapidly extracted from bacterial cells and then incubated with imipenem and phenol red [10, 13, 14]. This test demonstrated an excellent ability to detect different classes of carbapenemases (e.g., KPC, GES, NDM, VIM, IMP, and OXA-48) in *Enterobacteriaceae*, *Pseudomonas* spp. and *Acinetobacter* spp. [10, 13-16], though some concerns regarding the low sensitivity for OXA-48-like carbapenemase producers have arisen [17-20]. The test has also been directly implemented on positive blood cultures with carbapenemase-producing *Enterobacteriaceae* and *Pseudomonas* spp., demonstrating sensitivity and specificity >98% and 100%, respectively [21, 22]. Notably, the Carba NP test is recommended for the confirmation of carbapenemase production in Gram-negatives by CLSI [8]. Recently, this test became commercially available in an easy-to-use rapid kit (RAPIDEC Carba NP test; bioMérieux) [23].

The Blue-Carba test is another biochemical assay for carbapenemase production, using a different indicator (bromothymol blue) and a simplified protocol compared to the Carba NP test. The main advantage of this test is its faster TAT, since there is no need to extract the β -lactamase from colonies [24]. Overall, the Blue-Carba test shows comparable performance to the Carba NP test, but it seems to have better sensitivity, especially for

OXA-type carbapenemases [24]. A commercially available version (Rapid CARB Screen; Rosco Diagnostics) has shown similar sensitivity (97% vs. 98%), but superior specificity (100% vs. 83%), compared to the Carba NP test during an evaluation with *Enterobacteriaceae* producing different classes of carbapenemases [25]. In contrast, in another comparison the Carba NP test had sensitivities of 91% for *Enterobacteriaceae* and 100% *P. aeruginosa*, whereas those for the Rapid CARB Screen kit were 73% and 67%, respectively; the specificity of both tests was 100%. The cost of reagents for performing Carba NP test was 0.31 Euro and for CARB Screen was 1.25 Euro [26].

Single and multiplex end-point PCRs

The single PCR is advantageous if the detection of a single gene, such as KPC, is sufficient [27]. However, for specific gene characterization, DNA sequencing is frequently necessary (e.g., distinguishing SHV mutations with ESBL spectrum from those with non-ESBL activity). PCR also requires a high copy number of the target gene to produce a detectable amplification product [28]. Results can be obtained in <3-4 hrs for simple amplification to ≥ 24 hrs (for determination of DNA sequences). Examples of single PCRs designed to detect antibiotic resistance genes include detection of CTX-M [29, 30], TEM, SHV [31], KPC [32], IMP, VIM [33], NDM [34], Qnr [35], aminoglycoside modifying enzymes [36], and outer membrane porin genes [37].

The multiplex end-point PCRs use multiple primer sets allowing the simultaneous amplification of different targets in the same reaction [38]. Numerous multiplex PCRs able to detect resistance genes have been proposed. One paradigmatic example is the multiplex reaction designed by Perez-Perez and Hanson to detect the plasmid-mediated AmpC (pAmpC) genes with a total time from primary incubation to test results (TTR) of

about 3 hrs [39]. Voets *et al.* presented a design to simultaneously detect different families of ESBLs (SHV, TEM, CTX-M, GES, PER, VEB) [40]. Doyle *et al.* designed a multiplex reaction for the concurrent detection of KPC, NDM, VIM, IMP and OXA-48-like carbapenemase genes [41]. Cattoir *et al.* designed a multiplex reaction for the detection of different *qnr* genes [42]. Berçot *et al.* developed a protocol for the detection of 16S rRNA methylase genes [43].

Recently, Amplex Diagnostics has designed three commercial kits combining PCR and ELISA reactions to rapidly detect (2.5-4 hrs) *bla* genes. In particular, hyplex ESBL ID, hyplex SuperBug ID, and hyplex CarbOXA ID detect respectively: *i*) TEM, SHV, CTX-M, and OXA; *ii*) KPC, IMP, VIM, NDM, and OXA-48-like; and *iii*) OXA-23, OXA-24/40, OXA-51-like genes. Hyplex SuperBug ID showed a 97% agreement with the standard PCR/sequencing [44, 45]. In another study, the combination of hyplex ESBL ID and hyplex SuperBug ID was able to detect all class A and B enzymes among *Enterobacteriaceae* with high sensitivity (100%) and specificity (98%) [46]. Curetis AG has industrialized the Unyvero pneumonia assay, a fully automated multiplex PCR platform to identify 18 species and 22 resistance genes, including SHV, TEM, CTX-M, OXA, in ~4 hrs directly from respiratory samples [47]. However, comparison with conventional microbiological diagnostics showed poor performance of the assay [48, 49].

Single and multiplex real-time PCRs

The real-time PCR consists of an amplification reaction of the target gene coupled with the detection of the exponentially amplified DNA by monitoring fluorescence emission (e.g., with SYBR Green or TaqMan probes) [50, 51]. The real-time apparatus can also analyze the denaturing temperature (High Resolution Melting Analysis, HRM) of

synthesized DNA products, giving information on small variations (Single Nucleotide Polymorphisms, SNPs) in the sequence. The real-time PCR avoids time consuming steps such as running gels, and is more sensitive, reliable and cost-effective, and usually does not require DNA sequencing [52, 53].

Many in-house real-time PCRs for detecting ESBL and carbapenemase genes in Gram-negatives have been designed in the past [54-58]. Platforms to detect pAmpCs and plasmid-mediated quinolone resistance (PMQR) genes, or even perform multi locus sequencing typing (MLST)-like analysis, have also been developed [59-61]. These in-house platforms have also been extensively used to test clinical samples. For instance, Oxacelay *et al.* engineered a HYB probe for detecting CTX-M genes in urine [62]. Naas *et al.* engineered TaqMan probes for detecting NDM and OXA-48 producers in fecal specimens with detection limits of 10 CFU/100 mg and 10-50 CFU/100 mg, respectively [63, 64]. Two different TaqMan designs enabled detection of KPC genes directly from rectal swabs, showing superior sensitivity compared to selective cultures [65, 66]. One of these protocols was also adapted for detecting KPC genes within 4 hrs in blood culture bottles [67].

BioMérieux has developed a very rapid (< 2 hrs), highly sensitive and specific commercial real-time PCR kit (New NucliSENS EasyQ KPC) for the detection of KPC genes [68]. Check-Points Health BV provides a rapid (3.5 hrs) multiplex real-time PCR kit (Check-MDR ESBL) to detect SHV/TEM (distinguishing ESBLs from non-ESBLs) and CTX-M genes [69]. Similarly, the Check-MDR Carba kit rapidly identifies KPC, IMP, VIM, NDM, and OXA-48 genes [70]. The Check-Direct CPE assay is another commercial multiplex test that targets carbapenemases, and it has been recently evaluated

directly from rectal swabs: when compared with the selective culture approach, the sensitivity and specificity of the assay were 100% and 94%, respectively [71]. BD Diagnostics has developed a multiplex SYBR green real-time PCR coupled with HRM analysis for the detection of important carbapenemases in cultured isolates, showing both sensitivity and specificity of 100% [72]. Panagene designed a peptide nucleic acid (PNA) probe-based multiplex real-time PCR kits to detect carbapenemase genes, including KPC, OXA-48, GES, IMP, VIM, NDM, OXA-23, and OXA-58. For a collection of 318 Gram-negatives the sensitivity and specificity of the assay were >99%; the detection limit was of 100 copies/25 μ l reaction and the genes were all detected in <3 hrs [73]. GeneXpert (Cepheid) has developed the Xpert MDRO cartridge kit for detecting KPC, NDM, and VIM genes, requiring 2 min of hands-on time and 47 min to achieve results. High sensitivity and specificity (100% and $\geq$ 99%, respectively) have been recorded for fecal samples containing KPC or VIM producers [74]. Recently, an updated version (Xpert Carba-R test), designed for the detection of KPC, NDM, VIM, IMP, and OXA-48-like genes, has become available [75].

Loop-mediated isothermal amplification (LAMP)

By engineering the primers to form continuous loops rather than single bands, the LAMP method allows amplification and fluorescent detection at a constant temperature (no need for a thermo-cycler) of the target DNA [76]. This rapid and cheap method represents a promising alternative to PCRs and real-time PCRs. With in-house designs, Anjum *et al.* correctly amplified CTX-M and OXA-10-like genes from food samples [77], whereas Liu W *et al.* detected the NDM-1 gene in pure cultures as well as in sputum, urine, and fecal samples with a very rapid protocol (1 h) and with a detection limit of 10.7 pg/ml (which

correspond to 10^3 CFU/ml vs. 10^5 CFU/ml for the end-point PCR used as comparator) [78]. With a platform designed to detect KPC and NDM-1 genes, Solanki *et al.* showed that LAMP had greater sensitivity and specificity than end-point PCRs [79]. Nakano R *et al.* recognized homologous regions of all KPC genes at 68 °C in 25 min; the detection limit was 10 CFU/tube (10-fold more sensitive than PCR) both for pure DNA extracts and clinical samples (sputum, urine, feces and blood) [80]. Cheng C *et al.* developed a LAMP to detect KPC, NDM, IMP, and VIM carbapenemase genes. The lower detection limit was 10 CFU/reaction for real-time detection and 100 CFU/reaction for visual inspection [81].

The commercially available Eazyplex LAMP system (Amplex Diagnostics) is a qualitative genotypic diagnostic test consisting of a freeze-dried, ready-to-use kit. It was initially evaluated for the detection of carbapenemase enzymes (KPC, NDM, OXA-48, VIM, OXA-23, OXA-24/40, and OXA-58) in a collection of 82 well-characterized and non-clonally related *Acinetobacter* spp. strains. Using one colony, all isolates were correctly characterized in less than 30 min [82]. More recently, the Eazyplex SuperBug CRE kit has been evaluated to detect carbapenemases (KPC, VIM, NDM, OXA-48-like) and CTX-M-1/-9-like genes in previously characterized *Enterobacteriaceae* recovered in Spain. In this study, all carbapenemase and/or CTX-M producers were correctly identified in 15 min [83]. Extended kit versions able to further detect OXA-23-, OXA-24/40-, OXA-58-, and OXA-181-like genes are also available. The possible implementation of Eazyplex directly from clinical swab and without DNA extraction is promising but not yet validated.

Next-generation sequencing (NGS)

The Sanger methodology is still the most frequently used method for the sequencing of resistance genes. However, since this method is laborious, recent efforts have been made to overcome these limitations giving rise to the so-called “NGS” methodologies. These methods are based on the “real-time” fluorescence detection occurring during the sequencing process. Their commercialization promises to be revolutionary for the improvement of antimicrobial resistance determination because they are fast, reasonably cheap, and easy to interpret due to the development of bioinformatics tools [84-87].

An example of a NGS system is the bench-top 454 pyrosequencing system (GS Junior+, Roche) [88]. It has been used to discriminate the five CTX-M groups after real-time PCR by 13bp DNA region analysis [89], characterize carbapenemase GES variants [90], and distinguish chromosomal broad-spectrum SHVs from the acquired SHV ESBLs [91]. Moreover, the technology can also efficiently applied for the discernment of *aac(6)-Ib* from variant *aac(6)-Ib-cr* [92], and for the detection of *gyrA/parC* mutations conferring quinolone resistance [93].

Other promising NGS methods are also available. The sequencing by synthesis, commercially referred to as “Illumina”, consists of the neo-synthesis of DNA which is detected in real-time [86]. The sequencing by ligation, implemented by Life-technology with the commercial name “SOLiD”, is based on the action of a DNA ligase which adds fluorescent labeled dNTPs to an anchor probe hybridizing a single strand DNA [86]. The Ion torrent sequencing method (Life Technologies) is another powerful NGS approach based on the detection of the hydrogen ions released at each addition of a nucleic base [86]. Oxford Nanopore Technologies introduced two nanopore sequencing platforms capable of delivering high-throughput and ultra-long sequence reads at low cost [94]: the

GridION (for genome-scale sequencing) and the MinION (a disposable memory-key device that can be plugged into a laptop providing a gigabase of DNA sequence) use nanopores embedded within a synthetic membrane; as a DNA strand passes through the pore, each nucleotide induces changes in an electric current that can be translated into a DNA sequence [95].

Whole-genome sequencing (WGS)

In many studies, the WGS of single or relatively small numbers of interesting clinical isolates has been obtained. Genomic data are usually used to determine overall antibiotic resistance and virulence backgrounds [96, 97], evolution/adaptation under antibiotic selective conditions or diverse environments [98, 99], and clonal relationship of bacteria in different settings or geographic regions [87, 100].

For instance, Wright *et al.*, characterizing 49 *A. baumannii* isolates from one US integrated hospital system with the Illumina method, observed wide variations in gene content, even among strains that were almost identical phylogenetically. Transfer of mobile genetic elements (MGE), mobilization of insertion sequences, and genome-wide homologous recombination contributed to this diversity [101]. Performing WGS with Illumina for a collection of 103 *Acinetobacter* spp., Prichon *et al.* were able to detect 50 new OXA β -lactamases and 65 new *Acinetobacter*-derived cephalosporinases (ADCs) [102]. Snitkin ES *et al.* performed WGS with Roche 454 on longitudinal *A. baumannii* isolates from patients undergoing colistin treatment. Colistin resistance evolved via mutations at the *pmr* locus, but after colistin withdrawal susceptible strains were found, indicating a fitness cost preventing evolution to stable colistin resistance [98]. Holt *et al.* presented a detailed WGS analysis with Illumina HiSeq of 300 human and animal *K.*

pneumoniae isolates (including KPC and NDM producers) found in four continents. Strains could be split into three distinct species: *K. pneumoniae*, *K. quasipneumoniae*, and *K. variicola*. Human *K. pneumoniae* isolates had a large accessory genomes, including virulence functions associated with community-acquired invasive disease. In contrast, antimicrobial resistance genes were common in isolates colonizing patients or causing hospital-acquired infections [96].

Notably, the NGS methodologies can also be implemented to perform whole sequence analysis of plasmids carrying antibiotic resistance genes [103]. Thanks to the design and developed of supportive easy-to-use web tools (PlasmidFinder), this information has become of great importance to study the epidemiology, evolution, and clinical impact of plasmids [104]. The importance of these novel methodologies in re-defining the classes of epidemic plasmids has also been demonstrated [105].

Microarrays

Microarrays possess great diagnostic capacity because they can simultaneously detect and analyze a very large number of target genes [106, 107]. In the past, numerous in-house assays have been designed to characterize virulence, surface antigens, insertion sequences, plasmids and resistance genes. However, their implementation in a clinical laboratory is difficult because of problems related to the standardization of the procedures [108-112]. Recently, commercially available microarrays have solved this problem. These platforms are easy to perform and be updated, but the TAT is moderately high (6-8 hrs) and commercial kits are quite expensive [113, 114].

Check-Points has developed several automated DNA microarray to detect *bla* genes [107, 115-118]. One of these platforms (Check KPC/ESBL) was used to detect

319 ESBL and KPC genes directly in positive blood cultures, reducing the reporting time of
320 ESBL and/or KPC production by 18-20 hrs [119, 120]. Check-MDR CT101 assay, which
321 detects ESBL, KPC, pAmpCs, and NDM genes, showed 100% agreement with standard
322 PCR/DNA sequencing [114]. Check-MDR CT102, which detects ESBLs and the most
323 important carbapenemase (VIM, IMP, OXA-48-like, KPC and NDM) genes, showed an
324 agreement with PCR/DNA sequencing of 99% [121]. Check-MDR CT103 merged the
325 characteristics of CT101 and CT102, showing the same overall performances [106].
326 More recently, more clinically significant targets (i.e., OXA-23, OXA-24/40, OXA-58,
327 GES, GIM, SPM, BEL, and PER enzymes) have been added to the CT103XL assay to
328 obtain an advanced platform useful for the characterization of *Enterobacteriaceae*,
329 *Acinetobacter* spp., and *Pseudomonas* spp.

330 Alere Inc. prepared a large microarray assay containing probes to detect the virulence
331 factors of *E. coli* and further important resistance alleles (including CTX-M, KPC, NDM,
332 GIM, OXA-23, OXA-48, VIM, IMP, and Qnr) [113, 122-124]. This platform was
333 evaluated against a panel of 117 Gram-negative isolates, showing an overall 100%
334 concordance rate with the PCR method [125]. Nanosphere Technology has developed a
335 promising automated microarray (Verigene Nucleic Acid Test) for detecting bacterial
336 species and resistance genes within 2 hrs [126, 127]. In particular, the Verigene Gram-
337 negative Blood Culture Nucleic Acid Test is able to identify the most frequent Gram-
338 negative species along with CTX-M, KPC, NDM, VIM, IMP, and OXA enzymes.
339 Several authors evaluated its performance for positive blood cultures, obtaining
340 sensitivities and specificities of 87-90% and 100%, respectively [7, 126, 127].

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectroscopy
(MALDI-TOF MS)

The MALDI-TOF MS is nowadays often routinely used by clinical microbiologists to discriminate unique protein signatures (i.e., 16S ribosomal proteins) in order to identify bacterial species [128]. Overall, the main advantages of MALDI-TOF MS are its rapidity, very low costs, and consistency [129-132]. Recently, this system has also been applied to: *i*) recognize antibiotic degradation due to resistance proteins [133-142]; *ii*) discover mutations within resistance genes through DNA mini-sequencing [142]; and *iii*) study clonality of Gram-negative pathogens (e.g., detection of ST131 or ST405 *E. coli*) [143, 144].

Numerous studies have specifically assessed the utility of MALDI-TOF MS for the identification of β -lactam degradation products in the presence of hydrolyzing β -lactamases [137-141, 145, 146], including directly from positive blood cultures [147-150]. In particular, many studies have evaluated the identification of carbapenemase producers [132, 137, 138, 140, 146, 151]. Antibiotics (imipenem or meropenem) are incubated with the enzyme extract and then analyzed for degradation products with the MS; the time required to do this assay is about 1-4 hrs. However, we should note that this method can only detect the presence of β -lactam hydrolysis as a resistance mechanism and not the specific enzyme (e.g., distinguishing NDM from KPC) that can be determined with PCR- or microarray-based methodology. Therefore, the implementation of the MALDI-TOF MS for this aspect may be of limited value and this information can be easily obtained by implementing the rapid and cost-effective Carba NP and Blue-Carba tests [10, 16, 24]. On the other hand, MALDI-TOF MS possess higher sensitivity than the

Carba-NP assay due to its ability to better identify OXA or GES carbapenemase producers [152, 153].

PROMISING TECHNOLOGIES IN DEVELOPMENT

Rapid ASTs

Inhibition zone diameters around antibiotic disk can be continuously read using instruments consisting of a *real-time high-resolution video imager* in dedicated incubators. For instance, with the Advencis Bio-System instrument susceptibility to imipenem and production of ESBLs in Gram-negative bacteria can be detected in ~4 hrs [154]. This approach is also extended for use with micro-colonies under selective pressure or absence of antibiotics. A good example is the BACcelTM Digital Microscopy System (Accelerate Diagnostics) [5, 155].

The *ATP-bioluminescence method* provides a fast and reliable AST in 2-3 hrs. Testing both Gram-positive and Gram-negative strains, the sensitivity of this method compared to commercial systems (Microscan, Vitek) was 100% [156]. The method was also implemented to directly perform AST in urine samples, showing an overall accuracy of 91% [157].

The *cell lysis method* can be implemented to evaluate the activity of β -lactams against peptidoglycan. Cells incubated with the antibiotic are embedded in an agarose micro-gel on a slide, incubated in a lysis buffer, stained with a DNA fluorochrome and observed under fluorescence microscopy. When the bacterium is susceptible to the antibiotic, the debilitated cell wall is affected by the lysing solution, the DNA is released, and hybridized with a specific probes [158]. Similarly, the effect of quinolones on the chromosomal DNA can be evaluated [159]. Bou G. *et al.* combined these two techniques

to test a collection of *A. baumannii* strains for meropenem and ciprofloxacin susceptibility. All strains were correctly categorized in 100 min according to the MIC results interpreted with CLSI criteria [160].

The *flow cytometric analysis* can be used to detect ESBL producers. Cells are incubated with ceftazidime or cefotaxime for 1-2 hrs in presence or absence of clavulanate and then stained with a fluorescent dye able to diffuse across depolarized membranes. After incubation, non-ESBL isolates display increased fluorescence, whereas those producing ESBLs show this phenomenon only when incubated with clavulanate. The assay is rapid (~3 hrs) and correlated well with standard methods, but it cannot be performed with bacteria in stationary growth phase [161]. This principle was also implemented to perform a rapid AST (3 hrs) on positive blood culture bottles using the Sysmex UF-1000i flow cytometer. The essential agreement of the test with the commercial methods was 99% [162].

Numerous further potential AST configurations are under development. These near- or more-distant future technologies have been reviewed by other authors [5, 6, 163]. Among them, we should note that microfluidic methods are the most promising tools for obtaining rapid AST results (see below).

Microfluidics and nanotechnology

The rapidly evolving development of microfluidic and nanotechnology devices represents a basis for future applications in the laboratory diagnosis of antibiotic resistance. These lab-on-chip (LOC) miniaturized and automated diagnostics will become the point-of-care (POC) tests in the future, significantly affecting the outcome of patients [164-166]. These devices: *i*) use very small amounts of sample (from nano- to pico-liters) and reagents; *ii*)

incorporate multitasking functions in a disposable single cartridge performing different steps (e.g., cell lysis, hybridization, amplification); *iii*) do not need expensive equipment; and *iv*) assure high sensitivity, rapid results, and less work load for the operators due to innovative detection methods (e.g., optical, magnetic, electrochemical) [167-169].

Several authors have used microfluidic technologies to perform rapid AST for Gram-negatives. By observing the growth of *E. coli* in gas permeable polymeric microchannels, Chen *et al.* demonstrated that the large surface-to-volume ratio of microfluidic systems facilitates rapid growth of bacteria and analysis of antibiotic resistance profiles in 2 hrs [170]. Mach *et al.* implemented a biosensor-based AST device for urine samples. Growth of Gram-negative pathogens was determined in the presence of antibiotics in 2.5 hrs by electrochemical measurement of 16S rRNA, showing 94% accuracy compared to standard microbiological analyses [171]. Tang *et al.* used a microfluidic pH sensor to perform real-time rapid MIC determination. Confinement of bacterial cells in a nanoliter-size channel eliminates the need for long incubation (16-24 hrs), thus reducing the detection time to <2 hrs [172]. Choi *et al.* designed a microfluidic agarose channel chip that reduces the AST time to 3-4 hrs. The system immobilizes single bacterial cells by using agarose in a microfluidic culture chamber so that growth can be followed by microscopy time lapse imaging under different antibiotic conditions [173]. Kim *et al.* implemented a microfluidic device where continuous concentration gradients of antibiotics were generated to measure the minimal biofilm eradication concentration (MBEC) of *P. aeruginosa* [174]. Mohan *et al.* used a small volume (<6 μ l) microfluidic multiplexed platform that relies on fluorescence detection of bacteria for highly sensitive (one cell) and rapid ASTs (2-4 hrs) against *E. coli* [175]. Recently, Hou

et al. described a method to isolate low abundance pathogens (~100/ml) from whole blood using inertial microfluidics and directly determine species identification and resistance patterns in ~8 hrs with hybridization-based RNA detection [176].

Fluorescence in situ hybridization (FISH)

The FISH is a cheap, rapid, and easy to standardize methodology traditionally used to identify bacterial pathogens. In this context, it is based on the hybridization of short and specific (~18-25 bp) fluorescent-labelled, single-stranded probes to the rRNA target with subsequent analysis using fluorescent microscopes. The main advantage of FISH is its potential use directly from clinical specimens. This method has also been implemented to detect resistance genes, as confirmed for several commercial tests implemented for detecting clarithromycin resistance in *Helicobacter pylori* or *Campylobacter* spp. and presence of *mecA* gene in *Staphylococcus aureus* [6]. Palasubramaian *et al.* applied this approach to detect SHV variants with ESBL spectrum in positive blood cultures in ~1 h and with a limit of detection of 1.5×10^5 CFU/ml [177].

FilmArray

Idaho Technology (now BioFire Diagnostics) developed the FilmArray technique, a closed, very rapid (1 h) and fully-automated system that combines DNA extraction from clinical samples, nested multiplex PCRs, post-PCR amplicon melt curve analysis, and automated interpretation of results [178]. So far, this method has been applied to the detection of respiratory pathogens [179]. Recently, the FilmArray Blood Culture ID was evaluated in blood cultures to rapidly identify the causative pathogens and to detect several antibiotic resistance genes, including KPCs. However, data regarding the ability to detect KPC producers was not provided [180-182].

Rapid whole-genome sequencing (WGS)

In the past, it was commonly stated that the use of WGS for routine susceptibility testing was not practical due to its long TAT and the elevated costs compared with standard techniques [183]. Nowadays, the latest generation benchtop platforms (e.g., Illumina MiSeq and nanopore MinION) have almost eliminated these problems, making pathogen WGS data available to the diagnostic microbiology laboratory in less than a day for about 100 US dollars [184-186]. The challenge for the next few years will be the development of rapid and fully automated sequence interpretation software able to provide information (e.g., AST- and MLST-like results) understandable by laboratory workers and clinicians with no experience with WGS [187].

PCR/Electrospray Ionization Mass Spectrometry (PCR ESI-MS)

The PCR/ESI MS is a very promising technology that performs mini-sequencing-like of small PCR products (from 100 to 450 bp) by measuring their exact molecular mass and interpreting such data as a DNA sequence. This system has demonstrated success in the identification of pathogens and has been developed into a fully automated system (PLEX-ID; Abbott Biosciences) with results available within 4-6 hrs starting from clinical samples [129-131, 188, 189]. The rapid and fully automated ability to detect multiple genes (not only those conferring antibiotic resistance but also certain pathogens and virulence factors) in one reaction may represent a revolution for the diagnosis of infectious diseases [190]. Moreover, this platform offers the ability to detect pathogens at very low copy numbers and to determine genetic relatedness based upon an MLST-like approach [191-194], thus providing “real-time” data essential to detect outbreaks and limit spread of Gram-negative multidrug-resistant clones. Notably, the detection of DNA

from organisms that are non-cultured (for instance during antibiotic treatment) is a major step forward in the diagnosis of “culture negative infections” [195-198]. Unfortunately, PCR/ESI MS is still under development and currently only available for research applications at high cost.

The application of PCR/ESI MS for the detection of resistance mechanisms and SNPs within resistance genes in Gram-negative pathogens has been studied [188, 199]. Its utility for detecting KPC genes, evaluated using 110 well-characterized *Enterobacteriaceae*, indicated that the system correctly detected 100% of the KPC producers [200]. In another study, PCR ESI-MS quickly and accurately identified quinolone resistance mediated by mutations in the *gyrA* and *parC* genes of *Acinetobacter* spp. Single point mutations detected by PCR ESI-MS correctly correlated with susceptibility testing and DNA sequencing [201].

DISCUSSION

The development of resistance in Gram-negatives has challenged the Clinical Microbiology Laboratory to recognize the presence of these resistance mechanisms, appreciate their clinical significance, and develop methods to rapidly detect their presence. These challenges have been significant and in many instances difficult to address when conventional AST fails to recognize the presence of weak, but clinically significant resistance mechanisms such as carbapenemases and ESBLs. A further challenge has been to rapidly detect these resistance mechanisms in established cultures as well as directly from specimens. Review of methods to address these issues show the advances that have been made in rapid detection of resistance in cultures, but limited progress in direct detection from specimens.

There is also the inherent conflict between choosing between phenotypic and genotypic methods. Genotypic methods are rapid, can be used to test specimens as well as cultures, but are limited by the complexity of some targets and the evolution of new resistance mechanisms. Phenotypic methods are slow and best suited for use on cultures, but speed has been improved using microfluidics and progress is being made on direct specimen testing. It is likely that these challenges will continue as new resistance mechanisms emerge, and that both phenotypic and genotypic methods will continue to be needed and used.

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