The Evolving Role of the Clinical Microbiology Laboratory in Identifying Resistance in Gram-Negative Bacteria: An Update

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KEYWORDS

• AST • PCR • LAMP • MALDI-TOF • Sequencing • Rapid • Blood • T2MR

KEY POINTS

- Extensively drug resistant and pan–drug-resistant gram-negatives represent a global public health challenge.
- Rapid commercial phenotypic antimicrobial susceptibility tests now are available for laboratory use.
- Detection of resistance genes can be rapidly accomplished in cultures by immunoassays and nucleic acid amplification testing–based methods.
- Whole-genome sequencing directly on specimens is being developed for clinical applications.
- Advances have been made with direct detection of resistance genes from specimens.

INTRODUCTION

The clinical microbiology laboratory is challenged with detecting and characterizing antimicrobial resistance (AMR) in gram-negatives. Examples of recent and emerging resistance include the detection of extensively/pan–drug-resistant *Enterobacterales*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp producing carbapenemases (eg, KPC, NDM, and OXA types) together with other traits, such as 16S rRNA methylases and MCR, conferring resistance to aminoglycosides and polymyxins, respectively.[1–3]

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More rapid identification of AMR is a perpetual goal. Increased emphasis on rapid detection of resistance has focused on infections with the highest morbidity and mortality, in particular sepsis associated with bloodstream infections (BSIs). A mean decrease in survival of 7.6% for each hour after onset of infection until effective antibiotics are administered has been reported in sepsis.[4] Recent studies also have documented the value of more rapid resistance detection by the laboratory, which needs to be paired with more extralaboratory intervention. Rapid resistance detection has been shown to improve patient outcomes, with lower mortality, decreased hospital length of stay, lower superinfection and adverse drug reaction rates, and decreased costs.[5]

Although the rapid detection of bacteria and their resistance mechanisms directly from blood specimens is still a challenging target, this has been achieved on growing blood cultures (BCs), which typically become positive after 12 hours to 16 hours of incubation.[6] Many systems for rapid bacterial identification from positive BCs have been developed and, more recently, rapid automated antimicrobial susceptibility tests (ASTs) have been made available. Many of these systems also can detect AMR genes (ARGs).

AVAILABLE METHODS

Standard Antimicrobial Susceptibility Test 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 and Clinical and Laboratory Standards Institute (CLSI)[7,8] as well as regulatory agencies such as US Food and Drug Administration (FDA) and European Agency for the Evaluation of Medicinal Products. These organizations have established reference AST methods based on minimum inhibitory concentration determination by microdilution and agar dilution, with incubation times ranging from 18 hours to 48 hours. Disk diffusion methods also have been standardized.

Many commercial methods for AST are available and are based on using these methods directly or by methods providing comparable results. Commercial systems using reference microdilution methods include, for instance, MicroScan WalkAway (Beckman) and Sensititre (Thermo Fisher Scientific). Methods providing results comparable to reference testing include gradient diffusion minimum inhibitory concentration determination (Etest [bioMérieux] and MTS [Liofilchem]), and automated systems, such as Vitek (bioMérieux), Phoenix (BD Diagnostic Systems), and the rapid versions of MicroScan and Sensititre. Several of the methods have faster turnaround time (TAT) than reference methods, and those automated are coupled with machine-generated results. Instruments that record and interpret disk diffusion zone also are available (eg, ADAGIO [Bio-Rad]; Scan 1200 [Interscience]; and SIRscan [i2a]). Faster TAT also is available for disk diffusion testing using standard and enhanced media.[9,10]

Antimicrobial Susceptibility Test Methods to Detect Resistance Mechanisms

These reference AST methods include methods for determination of resistance mechanisms, such as (1) the presence of extended spectrum β-lactamases (ESBLs) using cefotaxime and ceftazidime alone and combined with clavulanate and (2) the presence of carbapenemases using lowered carbapenem breakpoints, the modified carbapenem inactivation method, and enzyme inhibitors (eg, boronic and dipicolinic acids).[7,11] These approaches are incorporated in many commercially available systems, such as those automated (eg, the Phoenix system)[12] or those based on disk diffusion (eg, the disk diffusion Neo-Rapid CARB kit [Rosco]).[13]

Rapid Antimicrobial Susceptibility Tests

The rapid AST systems include those based on flow cytometry; microfluidic; real-time high-resolution video imager; ATP bioluminescence; cell lysis; nanoechanical, electroechanical, and optomechanical; and other techniques (reviewed by Endimiani and Jacobs[14] and by Behera and colleagues[15]). Only several, so far, however, are available commercially. Q18

The Accelerate Pheno system (Accelerate Diagnostics) combines species identification (ID) through fluorescence in situ hybridization probes with rapid ASTs based on time-lapse automated morphokinetic cell microscopic analysis. Both ID and AST are performed automatically on positive BCs, with results provided in maximum 1.5 hours and 7 hours, respectively (at least 24 hours before those provided with routine approaches).[16] In a recent study, the system accurately identified the pathogens with a sensitivity ranging from 94.6% to 100%, whereas for the AST results, the categorical agreement was 97.9%.[17] Overall, the Accelerate Pheno system may significantly anticipate the definitive antibiotic therapy, improving the outcome of BSI patients.[18] Q19

The Alfred 60 (Alifax) is another automatic AST system implemented for positive BCs that provides results in approximately 6 hours. It analyzes the turbidity of bacteria that grow in broth and has demonstrated a 93% categorical agreement with the standard ASTs.[19]

Rapid Biochemical Tests to Detect Extended Spectrum β-Lactamase and Carbapenemase Producers

The ESBL NDP test is a rapid (15 minutes to 2 hours) and cost-effective biochemical test used to detect ESBL producers. 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, with reported 93% sensitivity and 100% specificity for detecting ESBL-producing *Enterobacterales* (ESBL-PE). The test has been evaluated on BC and urine samples, showing excellent sensitivity and specificity (>98% and >99%, respectively). This homemade test has been upgraded to a commercially available kit named Rapid ESBL NP test.[20] Q20 Q21

The Carba NP test is an in-house assay designed to detect carbapenemase producers. It detects a change in pH due to the hydrolysis of imipenem in presence of carbapenemases in less than 2 hours. β-Lactamases are extracted rapidly from bacterial cells and then incubated with imipenem and phenol red. This test demonstrated an excellent ability to detect carbapenemases in *Enterobacterales* and *Pseudomonas* spp, as well as in *Acinetobacter* spp, in an improved version (CarbAcineto NP test),[20,21] although there are concerns regarding the low sensitivity for OXA-48–like producers. The test also was implemented directly on positive BCs with carbapenemase-producing *Enterobacterales* (CPE) and *Pseudomonas* spp, demonstrating greater than 98% sensitivity and 100% specificity.[22,23] Notably, the Carba NP test is recommended for the confirmation of carbapenemase production in gram-negatives by the CLSI.[7] This test now is available commercially in an easy-to-use rapid kit (RAPIDEC Carba NP test [bioMérieux]). Another version of the original Carba NP test (Carba NP II test) includes additional wells with clavulanic acid and EDTA, making the assay able to distinguish the different classes of carbapenemases.[24] This test, however, is not commercially available.[20]

The Blue-Carba test is another in-house biochemical assay for carbapenemases detection, but it uses a different indicator (bromothymol blue) and a simplified protocol compared with the Carba NP test. The main advantage of the Blue-Carba is its faster TAT, because there is no need to extract the β-lactamase(s) from colonies. Overall, the

test shows comparable performance to the in-house Carba NP, with reported better sensitivity for the detection of OXA-type carbapenemases.[25] In a recent study with CPE, Carba NP had higher specificity than Blue-Carba (98.9% vs 91.7%, respectively), whereas both tests had 100% sensitivity.[26] A commercially available version of Carba NP (Neo-CARB kit, formerly Rapid CARB Screen) has shown similar sensitivity (97% vs 98%, respectively) but superior specificity (100% vs 83%) compared with the Carba NP test.[27] In contrast, in another evaluation, the Carba NP had sensitivities of 91% for *Enterobacterales* and 100% for *P aeruginosa*, whereas those for the Rapid CARB Screen kit were 73% and 67%, respectively; the specificity of both tests was 100%.[28]

β LACTA and β CARBA tests (Bio-Rad) are commercially available tests used for the detection of ESBL-PE and CPE, respectively. They rely on the use of chromogenic β-lactams that yield a different color when they are hydrolyzed by the β-lactamase (from yellow to red). Both tests are easy to perform and the results are obtained within 1 hour.[29] The β LACTA test has been evaluated not only with colonies but also directly from blood, urine, and bronchial samples. These samples yielded both specificity and sensitivity of 100%.[30] The β CARBA test showed high sensitivity (98%) and specificity (100%) in detecting CPE, including those producing OXA-48–like enzymes, from cultures.[31] Recently, it has been used directly on the pellet of positive spiked BCs: all CPE were detected and no false-positive results were recorded. Sensitivity and specificity were 100% and 94%, respectively, with TATs ranging between 20 minutes and 45 minutes.[32]

Biochemical Tests to Detect Other Resistance Phenotypes

The Rapid Polymyxin NP test (ELITechGroup) is a commercial assay that quickly detects polymyxin resistance quickly. This test is based on the detection of glucose metabolism related to bacterial growth (when resistant to polymyxins) in the presence of a defined concentration of colistin. The formation of acid metabolites is evidenced by a color change of the pH indicator red phenol in less than 2 hours. The assay showed greater than 98% sensitivity and greater than 94% specificity.[33,34] It also was evaluated for detection of colistin-resistant *Enterobacterales* directly from BCs, exhibiting excellent discrimination between colistin resistant and susceptible isolates.[35]

Based on the same principle used in the Rapid Polymyxin NP, further rapid phenotypic tests to detect aminoglycoside-resistant and fosfomycin-resistant *Enterobacterales* have been developed.[36,37] Because *Acinetobacter baumannii* and *P aeruginosa* do not metabolize glucose, a new assay (Rapid ResaPolymyxin *Acinetobacter/Pseudomonas* NP test) based on the utilization of resazurin (alamarBlue) has been developed. Metabolically active cells (polymyxin-resistant) reduce blue resazurin to the pink product resorufin. In less than 4 hours, the test showed 100% sensitivity and 95% specificity.[38]

Immunochromatographic Tests

Antigen detection can be used to detect enzymes or cell components of bacteria that are associated with AMR. The immunochromatographic tests often are lateral flow assays (LFAs) where antigen detection is identified by visualization of a line (as in pregnancy tests). These LFAs are useful because of their rapidity (results within 15 minutes), low cost, and accuracy that typically are comparable to nucleic acid amplification testing.[39]

The LFAs designed to detect β-lactamases started being commercialized in 2015 to 2016. Although at the beginning they were targeting only one enzyme (eg, OXA-48),[40]

nowadays multiplex LFAs are available. For instance, the RESIST-4 O.K.N.V. kit (Coris BioConcept) detects OXA-48–like, KPC, NDM, and VIM carbapenemases, with greater than 99% sensitivity and 100% specificity in culture strains belonging to *Enterobacterales* and *Pseudomonas* spp.[41] The NG-Test CARBA 5 (NG Biotech) detects the 5 most common carbapenemases: KPC, OXA-48–like, VIM, IMP, and NDM. Having shown 99.3% sensitivity and 99.8% specificity for cultured colonies,[42] this LFA now is FDA-cleared. Remarkably, CARBA 5 also has demonstrated high accuracy when testing positive BCs for detecting CPE (sensitivity and specificity of >97.7% and >96.1%, respectively).[43,44] An LFA to detect the colistin resistance traits MCR-1 also has been developed.[45]

Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectroscopy

The matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectroscopy (MS) nowadays is used routinely to identify bacterial species from growth on agar plates as well as organisms present in positive BCs. Overall, the main advantages of MALDI-TOF MS are its speed, relatively low costs, and consistency.[46]

Numerous studies also have assessed the utility of MALDI-TOF MS for the identification of β-lactam degradation products in the presence of hydrolyzing β-lactamases, including directly from positive BCs and urine. In particular, many investigators have evaluated the identification of carbapenemase producers where antibiotics (imipenem, meropenem, and ertapenem) are incubated with the organism and then analyzed for degradation products of the antibiotics with the MS; the time required to do this assay is approximately 1 hour to 4 hours.[47] Bruker Daltonics also produces the MBT STAR-Carba IVD commercial kit to rapidly detect carbapenemase producers. The assay showed high sensitivity (100%) and specificity (>98%) for CPE but not for OXA-23/-24–producing *A baumannii*.[48,49] All of these MALDI-TOF MS approaches, however, can detect only the presence of β-lactam hydrolysis as a generic resistance mechanism and not the specific enzyme (eg, distinguishing NDM from KPC); this identical information can be obtained easily by implementing rapid and cost-effective biochemical tests (discussed previously) or polymerase chain reaction (PCR)-based methods. The MALDI-TOF MS is able to detect the specific KPC-2 peak (28′544 m/z) in *Enterobacterales* and *P aeruginosa* with both sensitivity and specificity of 100%.[50]

Single and Multiplex Endpoint Polymerase Chain Reaction

A single PCR frequently is sufficient for detection of a unique ARG of interest. Subsequent DNA sequencing, however, may be necessary (eg, to distinguish SHVs with ESBL from those with non-ESBL spectrum). Results of PCR amplification can be obtained in less than 3 hours to 4 hours for simple amplification to greater than or equal to 24 hours if DNA sequencing is required. Making use of multiple primer sets, multiplex endpoint PCRs have the advantage of simultaneously amplifying many different targets. In the past, numerous single and multiplex PCRs have been designed to detect ARGs, including ESBL, carbapenemase, aminoglycoside-modifying enzyme, and outer membrane porin genes associated with carbapenem resistance (revised by Endimiani and Jacobs[14] and Lupo and colleagues[51]).

Single and Multiplex Real-Time Polymerase Chain Reaction

Real-time PCR consists of an amplification reaction of the target gene coupled with the detection of the exponentially amplified DNA product by various methods, such as monitoring fluorescence emission with SYBR Green or TaqMan probes. Real-time PCR avoids time consuming steps, such as running gels; is sensitive, reliable, and cost-effective; and usually does not require DNA sequencing. Modern

apparatuses also can perform a high-resolution melting analysis of DNA products, giving information on single-nucleotide polymorphisms in the sequence.[52]

Many in-house single or multiplex platforms for detecting plasmid-mediated AmpC (pAmpC), ESBL, carbapenemase, and other ARGs have been designed (eg, Endimiani and Jacobs[14] and Lupo and colleagues[51]), and many commercially available kits now are available. For example, Check-Points Health B.V. provides quantitative multiplex real-time PCR kits to detect ESBL and carbapenemase genes directly from peri/rectal swabs. Results are available within 2 hours to 3 hours, along with genotypic differentiation of the *bla* types based on probes labeled with different fluorescent dyes. Kits can be adapted to the BD MAX system (Becton-Dickinson), a diagnostic platform that operates as an open real-time PCR, allowing automated sample lysis, extraction, amplification, and detection processes. The Check-Direct ESBL screening kit detects CTX-M and SHV ESBL genes. For rectal swabs, it displayed sensitivity of 88% to 95% and specificity of 96% to 99%.[53,54] The Check-Direct CPE assay identifies bla_{KPC}, bla_{NDM}, bla_{VIM}, and $bla_{OXA\text{-}48\text{-}like}$, with reported sensitivity of 100% and specificity of 88% to 100%, respectively. Moreover, compared with standard approaches, this molecular system reduced TAT from 18 hours to 24 hours (using direct culture) or 48 hours (using broth enrichment) to only 3 hours.[55–58] For both Check-Direct kits, false-positive results (negative by culture) can arise from the presence of DNA residual of dead bacteria, or detection of bacteria harboring, but not expressing, *bla* genes.[53,54,56,58]

GeneXpert (Cepheid) is another real-time PCR system that performs fully automated nucleic acid detection and analysis directly from clinical samples. To minimize contamination, it is a cartridge-based, closed, self-contained platform. The company provides many cartridges for detection of different pathogens and ARGs. Among them, the Xpert Carba-R (v2) cartridge is designed to detect bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and $bla_{OXA\text{-}48\text{-}like}$, requiring 2 minutes of hands-on time and less than 48 minute to achieve results.[59] For rectal swabs, this kit demonstrated overall sensitivity of 97% to 100% and specificity of 99%. As for Check-Direct, the Xpert Carba-R assay reported the presence of carbapenemase genes in culture-negative samples.[60,61] In another study, Xpert Carba-R was implemented for rapid screening for colonization with carbapenemase-producing species, coupled with implementation of infection prevention strategies. Isolation of positive patients led to a reduction in both colonization (from 28.6% to 5.6%; $P<.05$) and infection (from 35.7% to 2.8%; $P<.05$) rates during the study period.[62]

Other companies have developed further real-time PCR-based platforms to detect carbapenemases, *mcr-1/-2* associated with polymyxins resistance and other ARGs. Examples include PANA RealTyper CRE kit (PANAGENE)[63]; Tandem-Plex CRE EU kit (AusDiagnostics)[64]; Acuitas AMR Gene Panel (OpGen)[65]; and GenePOC Carba/Revogene Carba C assay (Meridian Bioscience).[66] Their analytical performance directly on clinical samples, however, has not yet been extensively evaluated.

BioFire FilmArray

The BioFire FilmArray (bioMérieux) is a closed, very rapid (1-hour), fully automated system (only 2 minutes hands-on-time) that combines DNA extraction from samples, nested multiplex PCRs, post-PCR amplicon high-resolution melting analysis, and automated interpretation of results.[67] This method initially was developed for the detection of respiratory pathogens,[68] but later additional assays have been developed. The FilmArray Blood Culture Identification (BCID) kit has been approved by FDA for direct implementation on positive BCs. It identifies 27 targets, including gram-positives, gram-negatives, 6 *Candida* spp, and the ARGs *mecA*, *vanA/B*, and

304 bla_{KPC}. Similarly, the FilmArray Pneumonia Panel *plus* has 34 targets, including 27 ma-
305 jor respiratory pathogens and several ARGs (*mecA/C*, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP},
306 $bla_{OXA-48-like}$, bla_{CTX-M}, and bla_{KPC}).
307 The FilmArray BCID has been evaluated in numerous recent studies. In a large multi-
308 center trial (2207 samples), the system showed an identification sensitivity greater
309 than 96%. Moreover, sensitivity and specificity for *mecA* were both 98%, whereas
310 those for *vanA/B* and bla_{KPC} were both 100%.[69] In another study, it was shown that
311 the use of the BCID system reduced the time to optimal antimicrobial treatment in
312 ICU patients by an average of 10 hours (from 15 hours to 5 hours; $P<.05$).[70] Although
313 focusing on bacteremia due to gram-positives, another analysis showed that the
314 implementation of the BCID panel resulted in shorter postculture length of stay and
315 saved approximately $30,000 per 100 patients tested.[71] A new BC panel (BCID2),
316 able to detect further species and ARGs (including major carbapenemases and
317 *mcr-1*), will be released shortly.
318
319 ***Loop-Mediated Isothermal Amplification***
320 The loop-mediated isothermal amplification (LAMP) method allows amplification and
321 fluorescent detection of the target DNA at a constant temperature, avoiding the
322 need for a thermocycler. Genomic extraction from samples is not required as the ac-
323 tivity of the *Bst* DNA polymerase is not hampered by serum or heparin.[72] Recently,
324 many investigators have designed in-house LAMP platforms to detect different
325 ARGs. Overall, for clinical samples the LAMP was very rapid (<1-hour), more sensitive,
326 and with a lower limit of detection than PCR-based approaches.[14,73,74]
327 The commercially available eazyplex LAMP system (Amplex Diagnostics) consists
328 of a series of freeze-dried and ready-to-use kits coupled by real-time photometric
329 detection of amplified targets using the transportable Genie II instrument (OptiGene).
330 One of the kits was designed to detect KPC, NDM, OXA-48, VIM, OXA-23, OXA-24/40,
331 and OXA-58 carbapenemase genes. Its first evaluation was performed on *Acineto-*
332 *bacter* spp and all isolates were characterized correctly in less than 30 minutes.[75] In
333 another study focusing on *Enterobacterales*, an advanced kit (eazyplex SuperBug
334 CRE kit) was assessed to detect KPC, VIM, NDM, OXA-48–like, and CTX-M-1/-9–
335 like genes: all carbapenemase and/or CTX-M producers were identified correctly
336 within 15 minutes.[76]
337 The same kit also was used directly on 50 urine samples, 30 of which contained
338 ESBL producers; the assay showed sensitivity of 100% and specificity of 97.9%,
339 with results obtained in less than 20 minutes.[77] Recently, it was shown that implemen-
340 tation of the eazyplex SuperBug CRE kit on positive BCs significantly improved the
341 clinical outcome of BSIs due to CTX-M- and/or KPC/VIM-producing *Escherichia coli*
342 and *Klebsiella pneumoniae*. In particular, after notification of SuperBug CRE results
343 (on average 20 hours after sample collection), the proportion of appropriate treatment
344 increased from 6% to 71% and from 30% to 92% for BSIs caused by KPC/VIM and
345 CTX-M producers, respectively.[78] Extended kit versions able to further detect
346 pAmpCs (eazyplex AmpC), OXA-23–like, OXA-24/40–like, OXA-58–like, and OXA-
347 181–like (eazyplex SuperBug complete A/B/C and Acineto), IMI, GES, GIM (eazyplex
348 SuperBug expert), and the *mcr-1* (eazyplex SuperBug mcr-1) genes also are available.
349
350 ***Microarrays***
351 Microarrays possess great diagnostic capacity because they can simultaneously
352 detect and analyze a large number of target genes.[79] In the past, numerous in-
353 house assays have been designed to characterize ARGs, but their implementation
354 was difficult because of problems related to standardization of the procedures.

Recently, commercially available microarrays have become available. These platforms are easy to perform and can be updated readily, although the TAT is rather long (6–8 hours) and commercial kits are relatively expensive.[51]

Check-Points Health B.V. has developed an automated DNA microarray platform to detect the major *bla* genes. Over the past 10 years, several kits have been released, including Check KPC/ESBL, Check-MDR CT101, CT102, CT103, and CT103XL. Overall, these assays showed high accuracy in detecting ESBL, pAmpC, and carbapenemase genes in cultured strains.[80–82] Moreover, one of these kits (Check-KPC/ESBL) was used to detect ESBL and KPC genes directly from positive BCs, reducing the reporting time of these resistance traits by 18 hours to 20 hours.[83] The latest microarray kit made available by the company (New Check-MDR CT103XL) can detect the most epidemiologically important ESBL, pAmpC, and carbapenemase, along with the *mcr-1* and *mcr-2* genes. In a recent evaluation against a collection of *Enterobacterales*, all *bla* and *mcr-1/2* genes were correctly identified.[84]

Verigene System

Verigene (Luminex Corporation) is an automated multiplex microarray-based system that uses small aliquots of positive BC broths to identify a panel of major bacterial pathogens and ARGs. Results are available within 2.5 hours from Gram stain result on positive BCs. The test uses a disposable kit and cartridge, the latter inserted in a processor (5-minute hands-on-time) that carries out extraction of nucleic acid and microarray reactions. Final results are obtained by inserting the cartridge into a dedicated reader. Assays for gram-positives and gram-negatives are available. The Verigene gram-negative BC nucleic acid (BC-GN) test can identify *E coli*, *K pneumoniae*, *K oxytoca*, *P aeruginosa*, *S marcescens*, *Acinetobacter* spp, *Proteus* spp, *Citrobacter* spp, *Enterobacter* spp, and the ARGs bla_{KPC}, bla_{NDM}, $bla_{CTX\text{-}M}$, bla_{VIM}, bla_{IMP}, and bla_{OXA}. In a large study (1847 BCs), agreement of the BC-GN assay with the reference method for monomicrobial cultures was *E coli*, 100%; *K pneumoniae*, 92.9%; *P aeruginosa*, 98.9%; and *Acinetobacter* spp, 98.4%. Agreement for identification of ARGs was $bla_{CTX\text{-}M}$, 98.9%; $bla_{KPC/VIM/IMP}$, 100%; bla_{NDM}, 96.2%; and bla_{OXA}, 94.3%.[85]

Numerous studies also have demonstrated that implementation of Verigene BC-GN has a significant positive clinical impact. For instance, it was shown that ID (mean 10.9 hours vs 37.9 hours, respectively; $P<.001$) and time to effective therapy for BSI due to ESBL producers were achieved more quickly (mean 7.3 hours vs 41.4 hours, respectively; $P = .04$); moreover, length of intensive care unit (ICU) stay (12.0 days vs 16.2 days, respectively) and 30-day mortality (8.1% vs 19.2%, respectively) were significantly lowered.[86]

T2 Magnetic Resonance

The T2 magnetic resonance (T2MR) (T2 Biosystems) is a recently marketed system that combines PCR amplification, hybridization with nanoparticles and T2MR in a closed apparatus to detect diverse targets directly from complex matrices, such as blood.[87] With a limit of detection of 1 colony-forming unit/mL, the system can identify 5 *Candida* spp (T2Candida Panel) or *E faecium*, *Staphylococcus aureus*, *K pneumoniae*, *P aeruginosa*, and *E coli* (T2Bacteria Panel) from 2 mL of whole blood.[88] In ICU patients, T2Bacteria Panel showed sensitivity of 83.3% and specificity of 97.6% in detecting bacterial targets that were present in BCs. Sensitivity increased to 89.5% when patients with clinical indication of infection, regardless of BC results, were considered. A considerable number of patients, especially those receiving antimicrobials, had T2Bacteria-positive/BC-negative results. Mean times to detection of

species or negative results were 5.5 hours and 6.1 hours, respectively; in comparison, those for conventional BCs were 25.2 hours and 120 hours, respectively.[89] Recently, the company has developed a panel (T2Resistance) to rapidly detect 13 ARGs (bla_{KPC}, $bla_{NDM/IMP/VIM}$, bla_{OXA-48}, $bla_{CTX-M-14/15}$, $bla_{CMY/DHA}$, *vanA/B*, and *mecA/C*).

Next-Generation Sequencing

In the clinical setting, whole-genome sequencing of bacteria increasingly is used to inform on the emergence and spread of AMR, with the final objective to better tailor antimicrobial prescription.[90,91] No method other than pathogen genomic sequencing can deliver complete ARG and species identification directly from positive BCs or provide a full picture of the susceptibility profile as well as insights about novelty, transmission, and virulence of associated genetic elements. Genomic workflows typically involve several steps, from raw sequence data production to the further processing of the generated data into interpretable nucleic acid sequences using bioinformatic tools.

Over the past 15 years, the low-throughput, costly, yet accurate, Sanger sequencing has been replaced by high-throughput sequencing technologies, such as 454 pyrosequencing (discontinued in 2013) and Illumina sequencing. Currently, clinical genomic applications are based mostly on Illumina sequencing technology, which allows for the sequencing of entire genomes in mixed samples or the detection of sequence variants with enough coverage and with satisfactory base accuracy.[92] Although successfully used to profile human-associated antibiotic resistomes (eg, Forsberg and colleagues[93] and Gonzalez-Escalona and collaegues[94]), the short reads (few hundreds of bases) produced by the Illumina technology may lead to downstream sequence processing difficulties (eg, for contig assembly), especially when multiple copies of the same genes, high GC, or homopolymeric regions are present in the target genome.[95] Q28

High-quality de novo microbial genome assemblies can alternatively be obtained via Pacific Biosciences SMRT sequencing, which may produce sequences efficiently, even when long repeat regions are present.[96] Q29 The via Pacific Biosciences technology introduced in 2011, however, needs significant capital investment, dedicated personnel, and laboratory space, which may explain why only few applications have been reported in the clinical setting.[92] Consequently, clinically applicable workflows that provide straightforward, affordable, and comprehensive resistome characterization still are lacking, and technologies addressing those needs are highly desirable.

Oxford Nanopore Technologies introduced its first product, MinION, consisting of a single-molecule sensing system embedded in a cheap, light-weight (100-g) sequencer.[97] Nanopore sequencing works by threading individual DNA or RNA molecules through nanoscopic pores fixed to a membrane on which an ionic current is applied. As the molecule passes through the pore, the current is altered as a function of the identity of the base and of its residues. This signal then is recorded and converted into a nucleotide sequence by a suite of bioinformatic tools, while further processing of the data is done using software scripts provided by the company and by the user community. The strategy of Oxford Nanopore Technologies was to let a limited number of laboratories assess the sequencing performance of the device, acknowledging the developing nature of the technology. This early access to this technology has helped rapidly develop wet laboratory protocols, software scripts to optimize the sequencing process and also downstream analyses by a large group of users. It also lets users explore potential applications, thus contributing to publicize the new technology across a large array of scientific fields in a record amount of time. Nanopore reads are long, often reaching lengths greater than 100 kb,[98] and typically capture

entire genomic fragments, which facilitates downstream analysis of the genomic context when ARGs are identified.[99] This is significant particularly for clinical applications that aim at reducing TAT, particularly when a culture-independent, direct processing method to detect mixed microbial populations in samples is needed. In that respect, Cao and colleagues[100] demonstrated that bacterial species and strain information could be obtained within 30 minutes of nanopore sequencing based on approximately 500 reads, whereas initial drug-resistance profiles could be established in less than 2 hours, and complete resistance profiles could be available within 10 hours.

Whole-genome sequencing–based AMR predictions and antibiotic-resistance phenotypes often are concordant, with high sensitivity and specificity (>95%) reported for many phenotypes across several pathogen species,[101] although some notable exceptions were found, such as with levofloxacin resistance in *P aeruginosa*, where sensitivity and specificity may be below 95%.[102] Successful genomic applications in the context of bacterial drug-resistance characterization include the analysis of the structure and insertion site of an antibiotic resistance island in *Salmonella* Typhi[103] and the characterization of carbapenemase and ESBL genes in gram-negatives.[104,105] A functional metagenomics approach combined with nanopore sequencing was reported by van der Helm and colleagues[99] to characterize the resistome of clinical samples: clones from metagenomic expression libraries, derived from fecal samples obtained from an ICU patient, which could grow on each of a panel of 7 antibiotics, were selected, pooled, and barcoded with custom adapters and sequenced with the MinION nanopore sequencer. Resistome profiling identified a variety of ARGs with annotation accuracies of greater than 97% mean sequence identity, such as $bla_{\text{CTX-M}}$ and bla_{TEM}, genes coding for aminoglycoside-modifying enzymes, and diverse genes encoding ribosomal and efflux mediated resistance to tetracycline antibiotics.

Despite successful applications for strain identification and resistome profiling, emerging sequencing technologies that offer real-time, long-read, single-molecule sequencing of DNA or RNA molecules need further development in terms of (1) sensitivity, especially when applied to mixed samples, for which high-sequence yields providing sufficient genome coverage are required[100]; (2) sequencing accuracy to overcome the high error rate of the current nanopore sequencing technology (currently at approximately 4% per raw read), so that AMR-associated with mutations in chromosomal genes also can be identified[104] or multilocus sequencing typing schemes that attempt to identify bacterial strains from nanopore data be obtained reliably[92,100]; meanwhile, several postsequencing algorithms may be used to produce polished reads with accuracy greater than 98% to 99%; those algorithms include several rounds of mapping the raw reads to a consensus sequence in order to improve the overall consensus sequence quality[99,106]; (3) costs of flow cell and associated consumables[107]; and (4) easy-to-use bioinformatic tools and interfaces that facilitate the interpretation of the sequencing results by clinicians and that would enable a broader adoption of the technology in clinical settings in different countries.[108] Q30

Overall, single-molecule, real-time sequencing technologies, which may help better identify and characterize the genomic makeup of drug-resistant bacteria, have been shown not only to be technically feasible but also time and cost effective. Moreover, portable technology and rapid TAT provide actionable results with respect to infection control, implementation of personalized antibiotic treatment in high-risk patients, and on-site monitoring of resistome in both clinical and environmental settings. It is hoped that diagnostic laboratories soon will be able to implement routine genome sequencing as part of their surveillance programs for drug-resistant bacteria. Q31

DISCUSSION

The spread of extensively drug-resistant and pan–drug-resistant gram-negatives has challenged the clinical microbiology laboratory to recognize the presence of responsible resistance mechanisms, appreciate their clinical significance, and develop techniques to rapidly detect their existence. This overall challenge is significant and, in many instances, difficult to address when conventional AST fails to recognize the presence of clinically important resistance mechanisms, such as ESBLs and carbapenemases. A further challenge is to rapidly detect these resistance traits in established cultures as well as directly from specimens. This review shows the impressive advances that have been made in rapid detection of resistance in cultures (eg, positive BCs). Moreover, direct detection of ARGs from screening specimens (eg, rectal swabs) is a reality, whereas that from other primary samples (eg, whole blood) in the routine clinical context still is on the horizon.

There also is the inherent conflict between choosing between phenotypic and genotypic methods. Genotypic methods are rapid and can be used to test cultures as well as specimens but are limited by the complexity of the genetic targets and the continuing emergence of new resistance mechanisms. Phenotypic methods are slow and best suited for use on cultures, but speed has been improved significantly using rapid AST systems. It is likely that these challenges will continue as new resistance mechanisms emerge and that phenotypic and genotypic methods will continue to be needed and used in parallel.

DISCLOSURE

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Q8	This is how your names will appear on the contributors' list. **Please add your academic titles, if missing, as well as any other necessary titles and professional affiliations.** **ANDREA ENDIMIANI, MD, PhD, FAMH**, Institute for Infectious Diseases, Faculty of Medicine, University of Bern, Bern, Switzerland **ALBAN RAMETTE**, Institute for Infectious Diseases, Faculty of Medicine, University of Bern, Bern, Switzerland **DANIEL D. RHOADS**, Department of Pathology, Case Western Reserve University and University Hospitals Cleveland Medical Center, Cleveland, Ohio, USA

(*continued on next page*)

MICHAEL R. JACOBS, Department of Pathology, Case Western Reserve University and University Hospitals, Cleveland Medical Center, Cleveland, Ohio, USA

Q9 The following synopsis is the one that you supplied but edited down to less than 100 words. Please confirm OK or submit a replacement (also less than 100 words). Please note that the synopsis will appear in PubMed: 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. Recent development has enabled the use of novel methods for more rapid pathogen identification, antimicrobial susceptibility testing, and detection of resistance markers. Commonly used methods improve the rapidity of resistance detection from both cultured bacteria and specimens. This review focuses on the commercially available systems available together with their technical performance and possible clinical impact.

Q10 Please verify "gram-negatives", or eg, "gram-negative bacteria" or "gram-negatives infections", throughout.

Q11 Please check the hierarchy of the section headings.

Q12 If there are any drug dosages in your article, please verify them and indicate that you have done so by initialing this query.

Q13 If MCR is an abbreviation, please add expansion at first use.

Q14 Parenthesis count was mismatched in the paragraph. Please verify edit.

Q15 Please verify if manufacturers should include locations, throughout.

Q16 Please specify "These".

Q17 Please verify ESBL expansion, here and in heading below.

Q18 Please verify edits, for "These" — "The rapid AST systems".

Q19 Please verify FISH expansion.

Q20 Can NDP be expanded — "Nordmann/Dortet/Poirel"?

Q21 Please expand NP, or modify as NDP?

Q22 Please verify "Neo-CARB kit" vs "Neo-Rapid CARB kit", previously.

Q23 Add "high" before "specificity"?

Q24 Please verify edit, "false-positive results".

Q25 Please verify PCR expansion.

Q26 Verify edits, "Endimiani and Jacobs[14] and Lupo and colleagues[51]".

Q27 Please verify additions of "respectively" and verify that clear what 2 means refer to.

Q28 Is there an expansion for GC?

Q29 Expand SMRT ("single-molecule, real-time"?)?

Q30 Please verify if both "98%" and "99%;" are needed.

Q31 Does "time" mean "time effective"?

Q32 Please verify if both MSD and Merck Sharp and Dohme are needed.

Q33 Have we correctly interpreted the following funding source(s) and country names you cited in your article: Swiss National Science Foundation, United States; Oxford Nanopore Technologies, United Kingdom?

Q34 Please clarify the publication type (e.g., proceedings, journal, or book) in Refs. [8, 11].

(*continued on next page*)

Q35	Please provide page range for Ref. 9.
Q36	Please provide the volume number or issue number and page range for the bibliography in Ref. 19.
	Please check this box or indicate your approval if you have no corrections to make to the PDF file ☐

Thank you for your assistance.