Molecular diagnosis of antimicrobial resistance in Escherichia coli

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Review

Molecular diagnosis of antimicrobial resistance in *Escherichia coli*

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Abstract:

Introduction: Antimicrobial resistance is a growing global public health threat. The complexities of antimicrobial resistance in gram-negative bacteria such as Escherichia coli pose significant diagnostic and therapeutic challenges. Molecular diagnostics are emerging in this field.

Areas covered: The authors review the clinical importance of pathogenic E. coli and discuss the mechanisms of resistance to common antibiotics used to treat these infections. We review the literature on antimicrobial susceptibility testing and discuss the current state of phenotypic as well as molecular methodologies. Clinical vignettes are presented to highlight how molecular diagnostics may be used for patient care.

Expert commentary: The future use of molecular diagnostics for detection of antimicrobial resistance will be tailored to the context, whether hospital epidemiology, infection control, antibiotic stewardship, or clinical care. Further clinical research is needed to understand how to best apply molecular diagnostics to these settings.

Keywords: molecular diagnostics, mechanisms of resistance, Escherichia coli, antimicrobial resistance, whole genome sequencing, extended-spectrum beta-lactamase, carbapenemase, PCR, microarray
1.0 Introduction

The emergence and spread of antimicrobial resistance is a global crisis with profound health and economic consequences [1]. Drug resistant gram-negative bacteria are of particular concern worldwide given their substantial morbidity and mortality [2]. In this review, we will focus on antimicrobial resistance in the gram-negative bacterium *Escherichia coli*, which occupies central importance in this crisis because of its frequency as a gram-negative clinical isolate, its widespread carriage in the gastrointestinal tract of humans and animals, and its complexity of resistance mechanisms.

2.0 Clinical disease

*Escherichia coli* is arguably the most important single gram-negative pathogen of humans. It is the most common bacteria isolated in healthcare-associated infections (HAI) and leads to a spectrum of diseases that include catheter-associated urinary tract infections, central line-associated bloodstream infections, ventilator-associated pneumonia, and surgical site infections [3,4]. A Danish population-based cohort study of 14,000 episodes of bacteremia found *E. coli* to be the most common cause of community-acquired, nosocomial, and healthcare-associated bloodstream infections [5]. It is the most common cause of gram-negative bacterial sepsis [6-8] as well as one of the most common causes of neonatal sepsis and neonatal bacterial meningitis [9]. It is the major aerobic pathogen isolated in intra-abdominal
infections. Enterotoxigenic *Escherichia coli* (ETEC) is a significant etiology of travelers’ diarrhea, implicated in 20-40% of all travelers with diarrhea around the world [10,11], and *E. coli* O157:H7 is a leading cause of food-borne illness, hemorrhagic colitis, and hemolytic-uremic syndrome [12,13].

### 3.0 Global epidemiology of antimicrobial resistance in *E. coli*

Antimicrobial resistance among *E. coli* clinical isolates has increased significantly in the United States [14] and around the globe [15-17] over the past few decades. The rates of resistance vary by country and region of the world. In a 2016 study of neonatal sepsis in Delhi, India, the prevalence of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* clinical isolates was 47% [18]. Data from the 2007 Study for Monitoring Antimicrobial Resistance Trends (SMART) program in the Asia-Pacific region reported ESBL *E. coli* intra-abdominal infection rates of 79% in India and 55% in China, with significantly lower rates in Australia (8%) and the Philippines (17%) [19]. In the United States, the prevalence of ESBL *E. coli* pyelonephritis ranged from 3% to 12% from 2013-2014 [20]. Considerable regional variation is also observed in the fluoroquinolone resistance rates in *E. coli* urinary tract infections, ranging from 2% in Greece to almost 70% in India [21]. The prevalence of resistance to other antibiotics, like ampicillin and co-trimoxazole, varies by region as well [22]. Resistance mechanisms have also changed over time. In the 1990s, TEM and SHV were the predominant beta-lactamases and CTX-M producing organisms were found only sporadically around the globe. In the early 2000s, CTX-M producing isolates were noted more frequently in South America and then Europe, while still being rare in North America. Over the last 15 years, CTX-M enzymes have spread rapidly worldwide and are now the most prevalent ESBL [23].
The rapid global dissemination of CTX-M-15 is linked to the emergence and expansion of a high-risk bacterial clone, E. coli ST131 [24]. ST131 is a genetically fit clone that harbors several virulence factors that promote rapid dissemination, increased survivability, and successful horizontal gene transfer [25]. Within ST131, the most prevalent lineage is H30 (ST131-H30), the clonal expansion of which was responsible for a significant rise in drug resistant E. coli. In 2009, fully 22% of clinical E. coli isolates in the United States SENTRY Antimicrobial Surveillance Program were ST131-H30, and these isolates accounted for an excess proportion of fluoroquinolone, cephalosporin, and carbapenem resistant E. coli [26].

More details on resistance mechanisms will follow.

4.0 Mechanisms of antimicrobial resistance in E. coli

Bacteria can be intrinsically resistant or can acquire or develop resistance to antibiotics [27]. There are multiple mechanisms whereby bacteria can develop antimicrobial resistance, such as enzymatic alteration of the antibiotic, alteration or protection of the target site, restriction of access to the target, overproduction of the target, efflux pumps, decreased permeability, and others (Table 1). Frequently these mechanisms can be traced to one of three genetic processes: point mutations in chromosomal genes, acquisition of exogenous resistance genes via mobile genetic elements, or mutations in such acquired genes. These genetic changes then confer protein and functional changes that lead to phenotypic resistance via the mechanisms enumerated above [28]. We will now consider these processes for the major antibiotics used against E. coli.

4.1 Aminoglycosides
Mechanisms of aminoglycoside resistance in *E. coli* include enzymatic inactivation by aminoglycoside modifying enzymes (AME), efflux pumps, and alterations in the ribosomal target [28]. Aminoglycoside inactivation by AMEs is the major mechanism of resistance in terms of frequency and degree of resistance [29]. There are three types of AMEs: aminoglycoside acyltransferases (AACs), aminoglycoside nucleotidyltransferases or adenyltransferases (ANTs), and aminoglycoside phosphotransferases (APHs). These enzymes catalyze modifications at specific amino or hydroxyl groups, decreasing the ability of the drug to bind to the ribosome [30]. AcrD is a multidrug efflux pump that effluxes aminoglycosides from the cell [31,32]. Plasmid-mediated 16S rRNA methylases, like *rmtB*, affect the ability of aminoglycosides to bind to the ribosome via methylation of the aminoglycoside-binding site. These enzymes confirm high-level resistance to all aminoglycosides and are commonly found on plasmids carrying other drug resistance genes [33].

4.2 Beta-lactams

The major mechanism of beta-lactam resistance is via antibiotic modifying beta-lactamases. Chromosomal beta-lactamases are intrinsic to *E. coli*, however wild-type strains express these at low levels that are not phenotypically significant. Other beta-lactamases can be transferred via mobile genetic elements from one bacterium to another and can confer high levels of drug resistance [28]. Hundreds of beta-lactamases have been described [34]. There are two classification schemes for beta-lactamases. The Ambler system classifies enzymes based on their amino acid sequence into four major groups [35]. The Bush-Jacoby system classifies enzymes based on their functionality into three major groups and multiple subgroups [36].
Ambler Class A/Bush-Jacoby group 2 beta-lactamases include enzymes that hydrolyze penicillins and narrow-spectrum cephalosporins (TEM-1, SHV-1), extended-spectrum cephalosporins and monobactams (TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1) as well as carbapenems (KPC). These enzymes are generally inhibited by beta-lactamase inhibitors, with some exceptions [36].

Ambler Class B/Bush-Jacoby group 3 metallo-beta-lactamases use a zinc ion to hydrolyze the beta-lactam ring as opposed to Ambler Class A, C, and D beta-lactamases that contain a serine residue at their active site. Enzymes such as VIM, IMP, and NDM hydrolyze almost all of the beta-lactams including carbapenems, but do not have activity against monobactams [37].

Ambler Class C/Bush-Jacoby group 1 enzymes include AmpC beta-lactamases. Whereas these enzymes have activity against penicillins, they have particularly high affinity and activity against cephalosporins, including cephemycins and oxyimino-beta-lactams, and monobactams. Class C enzymes are resistant to inhibition by beta-lactamase inhibitors [38]. Wild-type \textit{E. coli} expresses low levels of chromosomally encoded AmpC enzymes constitutively. Overproduction of the chromosomal \textit{ampC} gene or acquisition of a plasmid-mediated \textit{ampC} gene results in higher levels of AmpC beta-lactamase production [39]. In \textit{E. coli}, the most commonly identified plasmid-mediated AmpC enzyme is CMY-2 [34]. Other examples include CMY-6, LAT-1, FOX-5, and DHA-1 [39].

Ambler Class D/Bush-Jacoby class 2 beta-lactamases can hydrolyze penicillins, with variable activity against some cephalosporins, monobactams, and carbapenems (OXA-48 variants) [40]. These enzymes are poorly inhibited by beta-lactamase inhibitors [36].

4.3 Fluoroquinolones
Mechanisms of resistance to fluoroquinolones include alterations in drug targets, reduction in drug accumulation through efflux pumps, antibiotic modification, and restriction of access to the drug target [41]. In general, high-level fluoroquinolone resistance occurs when multiple mutations are present in several genes [42].

The most common and clinically significant mechanism of resistance is via single point mutations in the target enzymes DNA gyrase and topoisomerase IV [28]. In gram-negative bacteria, DNA gyrase is more susceptible to inhibition by fluoroquinolones. Subsequently, resistance mutations commonly occur first in the DNA gyrase gyra gene [43], with mutations at positions 83 and 87 frequently associated with clinically significant resistance [44]. Common amino acid mutations in position 83 include Serine to Leucine or Tryptophan, and common mutations in position 87 include Aspartic acid to Glycine, Tyrosine, or Asparagine [45-47].

Alterations in drug target and efflux pumps often occur concomitantly in resistant E. coli isolates. For example, the AcrAB-ToLC efflux pump contributes significantly to fluoroquinolone efflux from the cell [48,49], and overexpression of this pump has been demonstrated in multi-drug resistant E. coli isolates [50]. An additional efflux pump that contributes to fluoroquinolone resistance is the plasmid-mediated efflux pump QepA [51].

Another plasmid-mediated quinolone resistance gene, aac(6′)-Ib-cr, encodes for a two amino acid substitution variant of the aminoglycoside acetyltransferase AAC(6′)-Ib protein [51]. The AAC(6′)-Ib-cr protein acetylates ciprofloxacin resulting in low level resistance to this antibiotic, whereas other fluoroquinolones are unaffected. This protein also confers resistance to several aminoglycosides [52]. Lastly, the plasmid-mediated quinolone resistance gene qnr encodes a protein that binds and protects DNA gyrase and topoisomerase IV from drug inhibition [53].
This mechanism is important for resistance to first generation quinolones (nalidixic acid) as well as ciprofloxacin [43,54].

4.4 Polymyxins

The most common mechanism of polymyxin resistance in *E. coli* is modification of the lipid A moiety of lipopolysaccharides (LPS) in the outer membrane of the bacterial cell, which is the primary target of the antibiotic colistin. This results in a net positive charge of the modified LPS, resulting in reduced drug affinity [55]. Efflux pumps also contribute to resistance, such as AcrAB-ToIC in *E. coli* [56]. In 2015, the *mcr-1* gene was identified as the first plasmid-mediated colistin resistance mechanism [57]. *mcr-1* has been isolated in various countries in Europe, Asia, North and South America as well as Africa and is frequently associated with other multi-drug resistance genes [58]. In 2016, *mcr-2* was identified in *E. coli* in Belgium [59]. Both *mcr-1* and *mcr-2* confer colistin resistance through modification of LPS [59,60].

4.5 Trimethoprim-Sulfamethoxazole

Acquired resistance to trimethoprim is mediated by two mechanisms in *E. coli*. The first is overproduction of chromosomal dihydrofolate reductase (DHFR), the target of trimethoprim, caused by promoter mutations. The second is the acquisition of transferable, low affinity *dhfr* genes (*dhfrI* and variants of *dhfrII*) [61]. Clinically, the plasmid-mediated trimethoprim resistant dihydrofolate reductases are the most important resistance mechanism, as both *dhfrI* and variants of *dhfrII* mediate high-level resistance that exceed achievable drug concentrations [62]. Acquired resistance to sulfonamides can occur through spontaneous point mutations in the chromosomal dihydropteroate synthase (DHPS), the target of sulfonamides, thus decreasing
susceptibility to sulfonamide inhibition [63]. Plasmid-mediated, transferable sulfonamide resistance is mediated by two genes encoding drug resistant variants of the DHPS enzyme, sul\textsubscript{I} and sul\textsubscript{II}. Sul\textsubscript{I} is frequently integrated into transferable plasmids with other resistance genes [62].

4.6 Macrolides

\textit{E. coli} is intrinsically resistant to low levels of macrolides due to decreased permeability of the outer cell membrane [64]. Acquired resistance can occur through three mechanisms. Modification of the macrolide target (23S rRNA) by methylases encoded by the \textit{erm}(B) gene results in decreased binding of the drug to its ribosomal target [65]. Multi-drug efflux pumps like AcrAB-ToIC [66] as well as a macrolide specific efflux pump encoded by \textit{mef} (A) [67] contribute to resistance. Lastly, drug inactivation occurs through phosphotransferases (\textit{mph} (A), \textit{mph} (B) genes) and esterases (\textit{ere} (A), \textit{ere} (B) genes) [68]. Whereas modification of the drug target results in broad macrolide resistance, mechanisms like efflux and drug inactivation only affect select macrolides [69].

5.0 Overview of antimicrobial susceptibility testing

A general understanding of the fundamental principles of antimicrobial susceptibility testing (AST) is a prerequisite to understanding the possible niche of new molecular diagnostics. The ultimate goal of AST is to predict the outcome of treatment with the antimicrobial agent tested [70]. Breakpoints, or interpretative criteria, are required so that the results of susceptibility testing can be interpreted as “susceptible,” “intermediate,” or “resistant”. The breakpoints are expressed either as a concentration (minimum inhibitory concentration or MIC) or zone.
diameter (in millimeters) [71]. These categories correlate with likely treatment outcome based on serum concentrations of antimicrobials. “Susceptible” suggests that antimicrobial agents will inhibit isolate growth when the recommended dosage is used. “Resistant” suggests that the antimicrobial agents will not inhibit isolate growth. The “intermediate” category may suggest cautionary drug use or that higher-than-normal drug dosage may be needed to ensure efficacy. This category also represents a buffer zone that prevents major errors in interpretations, such as categorizing a resistant strain as susceptible [72].

To establish a breakpoint, three main types of data are necessary: MIC distributions, pharmacokinetic/pharmacodynamic (PD/PD) data, and clinical outcome data [73]. Histograms or statistical descriptions of MIC distributions for an organism can help define wild-type populations and populations with resistance [74]. Clinical studies, when available, describe clinical and/or bacteriological outcomes as compared to the MIC for the organism. These studies have great value in evaluating cutoffs derived from MIC distributions and PK/PD data [71].

Two international standard setting organizations have published guidelines on the parameters used in setting breakpoints, the Clinical and Laboratory Standards Institute (CLSI) and the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST). The methods used by CLSI and EUCAST to derive breakpoints are slightly different, which results in occasional differences in the breakpoints between the two groups [75]. The CLSI interpretative criteria are developed using in vitro MIC data, PK/PD studies, and clinical outcome studies [76]. EUCAST interpretative criteria are established using an additional factor, the epidemiological cutoff values (ECOFFs) [73,77]. The ECOFF is defined as the MIC values that separate bacterial
populations into wild-type (no resistance) and non-wild-type (organisms with acquired or mutational resistance mechanisms) [77]. An important distinction is that ECOFFs are based on \textit{in vitro} data only.

6.0 Phenotypic methods of AST

Before describing the molecular methods for AST, we should elaborate on the phenotypic methods since there is considerable nuance to these methods. Additionally, such phenotypic AST will likely be the gold standard to which newer molecular technologies will be compared, since the approval of a new molecular diagnostic by regulatory bodies generally requires submission data that shows a major error rate of under 3% and a very major error rate of between 1.5 and 7.5% [78].

Reference methods for phenotypic testing can be divided into two categories: disk diffusion, which generates a zone diameter, and serial or gradient dilution, which generates an MIC [79]. Disk diffusion testing is technically simple and inexpensive. Antibiotic disk selection is flexible, and microbiologists are able to respond easily to changes in breakpoints. In general, zone diameter interpretative criteria correlate with MICs. However, in certain situations, even if the result is categorically susceptible, a quantitative MIC result indicating the degree of susceptibility may be helpful [79]. Additionally, there are certain drugs that do not diffuse well through agar and therefore give poor performance (ex. polymyxin). The Etest (bioMérieux, Marcy-l’Étoile, France) is a commercial phenotypic test that combines the simplicity of disk diffusion with the ability to estimate the MIC [80].
Broth microdilution is the phenotypic gold standard reference method [81]. Currently, four automated broth microdilution systems are FDA approved and commercially available in the United States: bioMérieux Vitek2 (Marcy-l’Étoile, France), BD Phoenix (Franklin Lakes, NJ), Beckman Coulter MicroScan WalkAway (Brea, CA), and Thermo Scientific Sensititre ARIS 2X (Waltham, MA) [82]. Results of automated antimicrobial susceptibility testing are generally available in less than 16 hours. Advantages of automated systems include reproducibility, ease of use, and rapidity of results. However, these systems can be significantly more costly than manual methods and are limited to antimicrobial panels that have been approved by the US Food and Drug Administration [83].

While the CLSI no longer recommend routine ESBL testing for clinical isolates, ESBL testing may still be useful for epidemiology or infection control purposes. The two methods detailed in the CLSI document are the combination disk method or a similar procedure using broth microdilution [76]. Both tests indirectly identify hydrolysis of a screening drug (cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone) by an ESBL enzyme by demonstrating improved activity of a confirmatory drug (cefotaxime or ceftazidime) in the presence of a beta-lactamase inhibitor [84]. The modified double disk synergy test (MDDST) is an alternative ESBL testing method. While this testing method may more reliably detect an ESBL when AmpC is co-produced, optimal disk spacing and result interpretations leave room for error in the absence of an experienced microbiologist [85].

Similarly, routine testing of clinical isolates for carbapenemase production is no longer recommended in the most recent CLSI document; however, identification of carbapenemase-producing *E. coli* remains important in infection control practices and epidemiologic
investigations [76]. The two tests described in the CLSI document are the Modified-Hodge test and the Carba NP test. The Modified-Hodge test is reported to accurately detect Ambler class A (KPC) and D (OXA-48) carbapenemase production, with a poor sensitivity (as low as 50%) for NDM-1 production [86]. The overall low specificity of this test (as low as 38.9% in one study [86]) is problematic [87,88]. The Carba-NP test accurately identifies KPC and NDM producers, but false-negative OXA-48 results are common [89]. The indirect carbapenem test is an alternative method that was shown in one study to have a comparable sensitivity but superior specificity to the Modified-Hodge test [90]. Lastly, a new phenotypic method for the detection of carbapenemase production in Enterobacteriaceae, the modified carbapenem inactivation method (mCIM), was reported in the literature to have an overall sensitivity and specificity of 97% and 99%, respectively. This test performed well with carbapenemase genes from Ambler classes A, B, and D [91].

7.0 Molecular methods of AST

Molecular detection of the antibiotic resistance markers enumerated above usually entails amplification and detection of DNA via PCR, microarrays, and/or genetic sequencing. Therefore, most molecular methods are limited by knowledge of which genes or mutations are known to be associated with resistance [92]. Other limitations are that the presence of multiple mutant genes or multiple gene copies is generally not captured, nor is the expression at the RNA or protein level, which can be critically important. Highly specific genotypic assays may be necessary, as illustrated by the example of polymorphisms near the active sites of either TEM or SHV enzymes altering the phenotypic expression from narrow-spectrum to extended-spectrum beta-lactamases [38]. Ultimately, the presence of a known resistance gene
(yes/no) is typically compared to a dichotomous (resistant/susceptible) phenotypic result, and this can lead to “false resistant” calls in phenotypically susceptible isolates should DNA be present but expression be low [93]. These nuances need to be considered within the intended context of use of the molecular diagnostic, whether for clinical use or epidemiologic/infection control purposes. Accepting these caveats, a number of molecular methods for AST are now emerging.

7.1 Real-time PCR

Real-time PCR is usually performed with sequence-specific primers and a fluorescently labeled internal probe and can be performed directly on a clinical specimen or a cultured isolate. Turnaround time for amplification and detection of targets is within hours. The number of unique fluorophores that can be used for simultaneous target detection, typically six or fewer, limits this technology. US FDA-approved commercially available examples of real-time PCR tests include the Xpert Carba-R (Cepheid, Sunnyvale, CA) and FilmArray blood culture identification panel (BCID) (bioMérieux, Marcy-l’Étoile, France).

Xpert Carba-R is a real-time PCR assay that identifies several carbapenem-hydrolyzing beta-lactamases, including KPC, NDM, VIM, IMP-1, and OXA-48. This rapid diagnostic test is approved for infection control purposes, to identify colonization by multi-drug resistant organisms from either pure culture or rectal swabs. Time to detection of resistance markers is less than one hour. Several studies have evaluated the performance of the Xpert Carba-R compared to phenotypic and molecular testing (PCR and/or sequencing) [94-98]. Across all organisms and genes detected, the sensitivity and specificity of the Xpert Carba-R was 93.5%-100% and 98.6%-100%, respectively, although the sensitivity to detect VIM was as low as 60%
in one study. Not all carbapenemases were equally represented in these studies, and the number of *E. coli* isolates tested was often not clearly delineated [97].

The FilmArray blood culture identification panel (BCID) is a multiplex PCR that detects a number of bacterial species as well as the carbapenem-hydrolyzing beta-lactamase KPC from positive blood cultures. This test is US FDA approved to aid in the diagnosis of bloodstream infections, with results available in one hour. One large multicenter trial evaluated the performance of the FilmArray BCID as compared to a targeted PCR assay for the KPC gene [99]. This study reported a 100% concordance between the two test methods, but as very few KPC-producing *E. coli* isolates were evaluated, further data are needed.

No real-time PCR assays for targets other than beta-lactamase genes are currently approved for clinical use. A number of non-FDA approved research-use only molecular diagnostics are available. The Check-Direct CPE kit detects KPC, OXA-48, VIM, and NDM (Checkpoints, Netherlands). The Antibiotic Resistance Genes Microbial DNA qPCR Array (Qiagen, Netherlands) detects beta-lactamase genes as well as aminoglycoside, macrolide, fluoroquinolone, and tetracycline resistance genes. The Eazyplex SuperBug kits (AmplexDiagnostics GmbH, Germany) detect beta-lactamase genes in addition to mcr-1. Lastly, Antibiotic Resistance TaqMan Assays (ThermoFisher Scientific, Waltham, MA) detect beta-lactamase genes and markers associated with macrolide and fluoroquinolone resistance. Available data on the performance of these tests is limited.

### 7.2 Microarrays
Microarrays can interrogate many more genes than multiplex real-time PCR methods, literally hundreds of targets, which is attractive given the diverse resistance determinants in gram-negative bacteria [92]. Limitations of microarrays include high costs, turnaround time, and inflexibility to adding new targets once an array is constructed.

The Verigene Gram-Negative blood culture nucleic acid test (BC-GN) (Nanosphere, Inc., Northbrook, IL) is a microarray-based test that identifies multiple bacterial species as well as many resistance genes from positive blood cultures within 2 hours. The targets detected by this assay of relevance to *E. coli* include the extended-spectrum beta-lactamase CTX-M and the carbapenemases IMP, KPC, NDM, OXA, and VIM. This test is US FDA approved to aid in the diagnosis of bloodstream infections. Several studies have evaluated the performance of the BC-GN test as compared to phenotypic susceptibility testing and/or confirmatory molecular testing (PCR and/or sequencing). Dodemont et al. [100] reported that CTX-M was correctly detected in 5 *E. coli* isolates, with 1 false negative CTX-M result. None of the other molecular targets were identified among the *E. coli* isolates. In Siu et al. [101], 34 CTX-M targets were identified in *E. coli* isolates and concordant with phenotypic testing. There were 4 false negative CTX-M results. No other resistance markers were identified in the *E. coli* samples. Other studies report a 100% agreement between BC-GN CTX-M identification and ESBL phenotypic susceptibility testing [102-105]. In a larger study, BC-GN detected 2 KPC genes and at least 1 IMP and VIM gene in *E. coli* isolates. Thirteen *E. coli* isolates were correctly identified as carrying NDM, and there were 2 false negative NDM results. As in the prior studies, CTX-M was the most commonly identified marker of resistance in the *E. coli* isolates [106].
Non-approved microarrays also exist. The Check-MDR arrays (Checkpoints, Netherlands) detect several beta-lactamase genes. The CarbDetect assay (Alere technologies GmbH, Waltham, MA) identifies beta-lactamase genes as well as aminoglycoside, macrolide, fluoroquinolone, and trimethoprim/sulfamethoxazole resistance markers. Lastly, the AMR-ve Genotyping kit (Alere technologies GmbH, Waltham, MA) detects beta-lactamase genes as well as genes associated with aminoglycoside, macrolide, chloramphenicol, tetracycline, fluoroquinolone, and trimethoprim/sulfamethoxazole resistance. Available data on the performance of these tests is limited.

7.3 Whole genome sequencing

Whole genome sequencing (WGS) offers even greater breadth of sequence interrogation since the entire genome of an isolate is sequenced. The cost of whole genome sequencing has decreased significantly in recent years and is now as low as $50 per isolate [107]. As a clinical diagnostic tool, several questions remain regarding the ability of sequencing to predict phenotypic resistance. Firstly, databases for resistance-associated mutations are still in evolution as our understanding of the underlying mechanisms of phenotypic resistance improves. As such, the sensitivity of detecting “known” mutations that are predictive of resistance is inherently incomplete since databases will only flag known mutations. There are technical issues as well, such as whether the WGS approach contains sufficient depth to screen resistance-associated genes. Finally, WGS is computationally complex and dependent on bioinformatics analysis algorithms that are also in evolution [108]. These bioinformatics challenges of WGS are becoming the rate-limiting step towards greater adoption. However, initial investigations are promising, particularly for *E. coli*. One study that included 50 porcine *E.*
coli isolates found that WGS, interpreted via the ResFinder analytical platform, revealed a 99.7% concordance with phenotypic testing using microdilution MICs [109]. This included phenotypic testing of a range of antimicrobial agents, including aminoglycosides, beta-lactams, macrolides, and sulfonamides. In general, these were relatively susceptible isolates without complex resistance patterns. One possible problem noted was the failure to detect streptomycin (an aminoglycoside) resistance. Another study from the UK used WGS performed on the Illumina HiSeq 2000 and analyzed against a custom study database of >100 known resistance associated loci. Among 74 E. coli isolates, there were 15 distinct genotypic resistance patterns and correlation against phenotypic methods (an automated BD Phoenix platform) revealed a sensitivity and specificity of 99% and 96%, respectively, for 7 antibiotics [107].

Another study from Texas evaluated whether WGS of E. coli isolates identified from bloodstream infections in neutropenic patients could predict resistance to ceftazidime, cefepime, piperacillin/tazobactam, or meropenem [110]. WGS was performed on the Illumina MiSeq and analyzed against a custom database built from the antibiotic resistance and comprehensive antibiotic resistance databases (ARDB and CARD). Of the 31 E. coli isolates tested, WGS as compared to the reference phenotypic testing method had a sensitivity and specificity of 93% and 97%, respectively, for resistance prediction among the four antibiotics.

For E. coli, most resistance to these antibiotics was related to CTX-M variants, followed by CMY and SHV-7 enzymes. There was no value to OXA-1 or TEM-1, which were detected but not associated with resistance to these antibiotics. One concern was the limited ability of WGS to detect piperacillin/tazobactam resistance (only 62% sensitivity), suggesting other mechanisms of resistance are operating or issues with gene copy number or expression may be playing a
role. However, the overall performance of WGS was similar to that of commercial MIC methods against the broth microdilution gold standard.

WGS studies are still in their infancy, such that the EUCAST Subcommittee has concluded that there is not sufficient evidence to support WGS for AST to guide clinical decision making at present [111]. Meanwhile, WGS is already in widespread use for other purposes, such as disease outbreak tracing and epidemiologic studies [109,112].

8.0 Clinical vignettes

8.1 Clinical scenario #1

An 87-year-old female with type 2 diabetes mellitus, chronic kidney disease, and a recent hospitalization for pneumonia requiring intravenous antibiotics presented to the emergency department from a nursing home with 2 days of pain with urination. She was febrile at time of presentation and had suprapubic tenderness on exam. Urinalysis was notable for numerous white blood cells and bacteria. The patient was admitted to the hospital and started empirically on ciprofloxacin (a fluoroquinolone) for the diagnosis of urinary tract infection. Blood and urine cultures were obtained at time of admission. The gram stain of the patient’s urine revealed gram-negative rods. On hospital day #1, the patient’s urine and blood culture grew a gram-negative rod, which was identified as *Escherichia coli*. Antibiotic susceptibilities were performed, and results available on hospital day #2 were notable for resistance to fluoroquinolones as well as aztreonam (a monobactam) and ceftriaxone (a cephalosporin). The isolate was confirmed to be an ESBL producing *E. coli*. In the interim the patient developed hypotension and required transfer to the medical intensive care unit. The physician reviewed
these results and decided to discontinue the ciprofloxacin and start meropenem (a carbapenem). After changing antibiotics, the patient’s fever, hypotension, and urinary symptoms resolved. Question: could molecular diagnostics have identified the resistance pattern sooner and improved this patient’s management?

8.2 Clinical scenario #2

A 45-year-old male with hepatitis C complicated by cirrhosis of the liver presented to his primary care physician with 3 days of abdominal pain and chills. He was referred to the emergency department after the physician noted fever, hypotension, abdominal tenderness, shifting dullness, and a fluid wave during the physical exam. In the emergency department, the patient was found to have an elevated white blood cell count and ultrasound imaging noted the presence of intra-abdominal ascites. Blood cultures were obtained. A diagnostic paracentesis was performed to sample the ascitic fluid, and laboratory findings were concerning for infection. Ascites fluid culture was obtained. He was started empirically on meropenem (a carbapenem). The initial gram stain of the patient’s blood and ascites fluid revealed gram-negative rods. On hospital day #1, the patient’s blood and ascitic fluid culture grew a gram-negative rod, which was identified as *Escherichia coli* in both cultures. Antibiotic susceptibilities were performed, and results available on hospital day #2 showed no drug resistance, including a favorable MIC to ampicillin (a penicillin). The patient’s symptoms improved, and on hospital day #2, meropenem was discontinued and ampicillin was started. Question: could molecular diagnostics have more rapidly optimized the antimicrobial use in this case, to decrease the unnecessary broad spectrum of activity of the carbapenem, and improved this patient’s management?
9.0 Expert commentary

Rapid molecular diagnostics targeting antimicrobial resistance genes have the potential to augment clinical care decision-making. This would likely require a microarray or WGS platform rather than individual real-time PCR assays due to the number of resistance genes that are of clinical importance. The optimal molecular diagnostic test would need a high positive predictive value (PPV) to warrant changing the antimicrobial agent if a drug resistance gene was identified. The early WGS results suggest such a PPV is possible. In clinical scenario #1, consider if a rapid molecular diagnostic test had been performed at the time of bacterial culture. If the molecular test identified resistance determinants within a few hours, and if the test had a high PPV, the antibiotic could have been changed to an effective agent earlier in the patient’s clinical course. This process could be expedited further if the test could be run directly from a clinical specimen (such as the urine sample) rather than a cultured bacterial isolate; however, for non-sterile sites, attributing a particular resistance marker to pathogenic *E. coli* rather than other commensal flora may be hazardous.

The optimal molecular diagnostic test would need a high negative predictive value (NPV) to warrant narrowing the spectrum of antibiotic activity if no resistance markers were identified. In clinical scenario #2, consider if a rapid molecular diagnostic test reported no detected resistance markers, signifying a wild-type bacterial strain. If the test had a high NPV, then the antibiotic could have been changed earlier in the patient’s clinical course. It should be noted that the number of assays needed may be so broad that they may be impractical, and may need to be adapted for local resistance mechanisms.
At present, therefore, we feel that standard phenotypic AST is still necessary. In multi-drug resistant infections, knowledge of the full susceptibility profile informs complex decision-making regarding antibiotic selection as well as alternative agents if side effects develop to the drug. Some infections require several weeks to months of antibiotic therapy, such that the full phenotypic AST profile with CLSI breakpoints remains essential to optimal drug selection with the greatest efficacy and least toxicity. The MICs to antibiotics with excellent oral bioavailability can influence whether oral antibiotics can be used rather than intravenous antibiotics in certain clinical scenarios. Lastly, in some serious infections, MIC values influence whether multiple drugs are needed for treatment.

That said, the use of molecular diagnostics is becoming more important as new antibiotics are developed. Two novel beta-lactamase inhibitors with activity against *E. coli* have been recently US FDA approved. Ceftazidime-avibactam has shown activity against class A (KPC) and some class D beta-lactamases [113]. Meropenem-vaborbactam has shown activity against class A (KPC) beta-lactamases [114]. Additional novel beta-lactamase inhibitors are in development at the time of this review. As these drugs have activity against some beta-lactamases but not others, documenting the molecular mechanisms of resistance will be key for optimal use of these antibiotics.

**10.0 Five-year view**

As the global antimicrobial resistance crisis heightens, clinicians will encounter more infections caused by antibiotic resistant organisms. Molecular diagnostics for gram-negative bacteria like *E. coli* will have an important role in the future but performance will need to be better understood and their application carefully considered.
Infection control and surveillance protocols within hospitals or healthcare systems will increasingly utilize molecular diagnostics to prevent the spread of multi-drug resistant organisms (MDRO). Simple, real-time PCR tests on direct patient specimens, like rectal swabs, for MDROs make sense in this context as they provide rapid results for selected targets of interest. This information feeds back into infection control policies and practices, like the use of contact precautions (gown, gloves) by healthcare providers when examining patients colonized by a MDRO organism.

For molecular epidemiology, WGS will likely become the primary modality as it provides unprecedented insight into the mechanisms of resistance as well as bacterial strain typing. As mentioned previously, bioinformatics pipelines are the limiting steps to widespread implementation of this technology.

Antimicrobial stewardship entails a multifaceted strategy to improve the use of antimicrobials and reduce antimicrobial resistance rates. Molecular diagnostics, whether real-time PCR, microarrays, or WGS, have the potential to contribute to this field.

Regarding application of molecular diagnostics for routine clinical care, we believe that when considering *E. coli*, a qualitative (susceptible/resistant) result from WGS or microarray data should become technically possible in a 5-10-year time frame. The rate-limiting steps will be turnaround time with bioinformatics pipelines and how important it will be for clinicians to know the exact MIC, a level of resolution that will be difficult for molecular AST to provide. All of the foundational guidelines detailed earlier in this review for determining antibiotic susceptibility and resistance are based on PK/PD and MIC data, and it is difficult to envision this being superseded soon. Rapid phenotypic tests will also compete with rapid molecular
diagnostic tests for use in clinical care. While it seems phenotypic AST will remain common practice, the molecular methods and databases will certainly enlarge and improve molecular diagnostic performance in the near future. Clinical trials that compare outcomes with different diagnostic algorithms (molecular versus phenotypic) will become important, particularly if these include not only patient-specific but also antimicrobial stewardship and infection control outcomes.

**Key issues**

- Antimicrobial resistance is a global threat that stands to get worse.
- Molecular diagnostics to detect drug resistant organisms such as *E. coli* will continue to improve and evolve, but they must interrogate several molecular targets and be compared against complex phenotypic AST methodologies.
- Simple real-time PCR methods offer simplicity and fast turnaround time, whereas WGS methods offer breadth but require sophisticated bioinformatics pipelines. Microarray methods are intermediate.
- The application of molecular diagnostics for infection control and epidemiology are clear, whereas their use for clinical management and antimicrobial stewardship will require further clinical research over the next several years.

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Declaration of Interest

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References

Reference annotations
* Of interest
** Of considerable interest


**Economic analysis that put the AMR crisis on global terms, predicting that by 2050 AMR may cause 10 million deaths/year, more than cancer, and cost up to 100 trillion USD in lost GDP


** A large cohort study that reported an alarming incidence and mortality of multidrug resistant gram-negative bacterial sepsis in neonates born in tertiary care hospitals in India


* Thoughtful commentary on the emergence and worldwide dissemination of CTX-M-producing isolates


** Comprehensive review of the emergence of high-risk clones and its impact on the antimicrobial resistance crisis worldwide


77. The European Committee on Antimicrobial Susceptibility Testing [Internet]. Available from: http://www.eucast.org


* Thorough review of the complexities and challenges of molecular antimicrobial susceptibility testing


** A promising study that shows WGS as a potential molecular tool for prediction of antimicrobial resistance in clinical isolates, with thoughtful consideration of limitations


* Smaller study of clinical *E. coli* isolates but captures the appeal of WGS as compared to PCR and conventional phenotypic susceptibility testing methods in a high risk patient population


<table>
<thead>
<tr>
<th>Antibiotic Class</th>
<th>Mechanism of Action</th>
<th>Mechanism of Resistance</th>
<th>Enzymes</th>
<th>Genes</th>
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<td>Inhibit protein synthesis</td>
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<td>Target modification</td>
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<td>Phosphorylases</td>
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Table 1. Molecular mechanisms of resistance to key antibiotics against Escherichia coli
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