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Commentary

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Antibiotic susceptibility testing for therapy and antimicrobial resistance surveillance: genotype beats phenotype?

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"Accurate AST has become essential to improve antimicrobial stewardship and clinical outcomes under the dramatic scenario of antimicrobial resistance."

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Bacterial diagnostics combines species identification and antibiotic susceptibility testing (AST). Diagnostic companies offer laboratory automation solutions that execute sample preparation, sample processing, isolates identification, species determination and AST. Matrix-assisted laser desorption/ionization-TOF mass spectrometry (MS) has almost replaced phenotypic species identification and is the gold standard method nowadays. Despite a comparably high level of automation, AST is still performed by classical microbiological methods like agar diffusion (disk diffusion) and broth (micro)dilution, which are the workhorses of automated and manual AST (variations like gradient strip tests included). International norming bodies like the Clinical and Laboratory Standards Institute and the European Committee for AST define procedures by using technical ISO standards and regulate how AST has to be performed in diagnostic laboratories worldwide [1,2]. National regulations demand adaptations to these norms due to licensing, reimbursement and accreditation requirements.

Over the years and decades, a huge armamentarium of various additional phenotypic and genetic AST methods has been developed based on an increased understanding of underlying antibiotic resistance mechanisms. As part of the aforementioned norming and legal requirements, some of these additional phenotypic and genetic tests are required to confirm or specify classical phenotypic AST results. For instance, oxacillin resistance in methicillin-resistant *Staphylococcus aureus* (MRSA) requires confirmation of a simple AST result by (real-time) PCR for the corresponding *mecA* or *mecC* genes or latex agglutination assays detecting the expressed PBP2a protein. Advanced phenotypic tests, as for instance, introduced to diagnose and differentiate the great variety of extended spectrum beta-lactamases and carbapenemases in Gram-negative bacterial pathogens, may be sufficient and even advantageous for a reliably fast diagnostic. In the case of an invasive infection and the suspicion of a carbapenemase-producing Enterobacterales isolate, a fast and reliable phenotypic assay may be the first choice over classical but time-consuming AST methods. Fast immunochromatographic or colorimetric tests or a real-time PCR generate a putative result in up to 1 h or at least much faster than automated AST [3,4]. In other instances, a positive test result of an additional phenotypic or genetic test may overrule a negative classical AST result, for instance, in case of a weak expression of the corresponding determinant of interest. Rather than replacing phenotypic susceptibility testing, nowadays genotypic testing complements it.

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Accurate AST has become essential to improve antimicrobial stewardship and clinical outcomes under the dramatic scenario of antimicrobial resistance. In this commentary, we provide a scientific-based opinion about current essentialities and constraints of phenotypic AST under the different scenarios of clinical diagnostics and surveillance.

Advantages & drawbacks of phenotypic AST

Routine antibiotic susceptibility testing (AST) usually takes a minimum of 24 h to obtain growth of bacterial colonies and an additional 18–24 h to obtain isolate characterization (biochemical identification and phenotypic AST). Advantages of AST include the prediction of resistance or susceptibility with a high degree of standardization and in a quantitative manner. Classical manual methods (e.g., disk diffusion) are time-consuming with semi-automated or automated broth microdilution systems (e.g., Phoenix, Vitek 2, Microscan) reducing incubation and hands-on times (6–12 h) since the 1970s [5]. However, such systems also have limitations that include a complex pre-analytical process, the need for viable bacteria (low sensitivity), the limited bacterial spectrum in the database and the costs. In fact, the development of faster AST did not parallel all technological advances in identification, thus limiting the potential effects of early pathogen identification.

The desirability for quick results and the technical achievements in recent years opened the doors for some high-volume data and high-throughput technologies to become more and more developed into automated AST prediction. Whereas transcriptomics- and proteomics-based technologies are mainly still in the experimental phase [6], genome-based (including metagenome-based) prediction of AST seems quite advanced [7].

Susceptibility & resistance determination to guide a therapeutic decision

In the first instance, antibiotic susceptibility testing (AST) is performed to guide antibiotic therapy and patient treatment. Especially in the case of a fast-progressing and life-threatening invasive bacterial infection, time-to-result is essential as it will directly influence survival and death. According to many national and international guidelines and regulations, classical or automated phenotypic AST is legally required. As explained already, genetic or additional phenotypic tests may complement but not substitute classical phenotypic AST. When performing AST by, for instance, broth microdilution, the most important value is the MIC of a bacterial isolate, meaning the lowest concentration in a series of doubling dilutions of an antibiotic that inhibits the visible growth of the investigated isolate. For classical phenotypic assays, the final clinical orientation is based on the breakpoints set by corresponding authorities like Clinical and Laboratory Standards Institute (CLSI) or European Committee for AST (EUCAST) who face an ongoing challenge to provide optimal reports for the best therapeutic decision. To add uncertainty, these agencies use different criteria and publish different clinical breakpoints for the same antibiotic. These breakpoints were set as 'clinical breakpoints' since they also consider pharmacokinetic/pharmacodynamic parameters, dosing regimens and serum levels achievable and required to treat and eradicate a corresponding bacterium [8]. In the simplest way, a clinical breakpoint may differentiate into 'susceptible' and 'resistant', highlighting the proposed success or failure of an intended therapy by the given antibiotic, but in practice it is quite often not as simple as that [9]. For many resistance phenotypes, the underlying resistance mutation or the acquired resistance gene(s) are known, making a genetic or genomic prediction of AST technically possible [10]. However, a number of obstacles have to be considered, before a 'clinical breakpoint' and thus a putative therapeutic success from a genetic or genomic result could be predicted or derived. Phenotypic or classical AST measure the expression of a given gene, meaning the activity of an enzymatic degradation or other detoxication of the antibiotic, an increased release or decreased entry into the cell, among other mechanisms. A genetic or genomic result determines gene presence or absence or resistance mutations associated with a given phenotype. For 'classical' antibiotics many associations are simply transferable, when well-known resistance genes and mutations for certain determine a given phenotype. Examples are manifold, for example, MRSA detection by *mecA* gene presence measurement, or a very well-established genetic resistance prediction in slow-growing mycobacteria, especially *Mycobacterium tuberculosis* (*rpo*B mutations predict rifampicin resistance).

Already 2 decades ago, EUCAST introduced the concept of epidemiological cut-off values (ECOFFs) as an attempt to infer resistance trends in real-time, taking advantage of routine/surveillance susceptibility testing results. ECOFFs define the MIC above which bacteria have phenotypically detectable acquired resistance mechanisms, meaning unnoticed or novel resistance genes may be identified when applying such ECOFFs. However, ECOFFs are not applied in the healthcare context, nor in many epidemiological contexts where whole-genome sequencing is accessible and advantageous as it provides the entire bacterial genetic makeup. While lacking affordable and easy

sequencing, the identification of non wild-type strains by clinicians could be an alert for specific situations where phenotypic AST evaluated by clinical breakpoints does not guarantee a successful therapy. In fact, different studies already corroborated a strong correlation between ECOFFS and genetic determinants [11]. At least for surveillance purposes, ECOFFs could be included in official reports and platforms (e.g., ECDC Surveillance Atlas) to promptly anticipate emerging resistance threats.

However, it is not all straightforward in the clinical routine. Some AMR genes are expressed at low levels below clinical breakpoints and other deviations in detection may occur as well among leading hospital bugs. A relevant example is vancomycin-resistant enterococci (VRE) carrying *vanB* genes expressing low-level (inducible or not) resistance or carrying *vanA* genes but not expressing clinical resistance at all ('vancomycin-variable enterococci', [VVE]) [12]. Notably, VanB-VRE types have been linked to vancomycin-susceptible outbreak isolates. Both VanB-VRE and VVE are increasingly reported across Europe, often not detected properly by routine phenotypic screening methods and automated systems, leading to pitfalls in routine VRE detection and an unnoticed transmission of *van* genes. Also, in enterococci we increasingly observe hospital strains that carry acquired and mobile resistance genes (*optrA, cfr, poxtA*) expressing 'borderline' or 'low-level' resistance to linezolid, meaning the MICs alternate between 4 (S, susceptible) and 8 (R, resistant) mg/l and thus along the clinical breakpoint of >4 mg/l [13]. The clinical consequences of these diagnostic uncertainties are unknown since large scale studies are non-existent with rare enterococcal isolates harboring resistances to antibiotics of last resort. Failure in the detection of MRSA is also possible in the presence of sporadic divergent homologs of *mecA* or (SCCmec)-cassette subtypes, when MRSA carriage is below detection limits of commercial tests or when genetic tests fail to amplify corresponding *mec* resistance genes in otherwise phenotypically resistant staphylococci [14].

Despite the vast arsenal of methods for carbapenemase-producing Enterobacterales detection, they can still evade common diagnostic screening systems. In particular, OXA-48 and some VIM producers are hardly detectable by common automated AST systems [15]. This phenomenon is being increasingly associated with hard to control outbreaks and the spread of strains or plasmids carrying $bla_{\text{OXA-48-}}$ or similar bla_{OXA} genes like $bla_{\text{OXA-244}}$ that might express variable levels of carbapenem insusceptibilities [16]. Another diagnostically challenging situation may result from Enterobacterales with colistin resistance, which is due to mobile *mcr* genes. Different studies reported *mcr-1*-carrying isolates showing MICs of 2 mg/l, which is below the EUCAST/CLSI clinical breakpoints (>2 mg/l) and should be considered in targeted screening approaches requiring further genotyping confirmation [17].

There are additional obstacles in phenotypic or genetic AST determination and genomic AST prediction. Antibiotics of last resort are a valuable option to treat multidrug- and extensively drug-resistant hospital pathogens. Although not that widely disseminated, resistances to drugs of last resort also emerged and spread. Phenotypic AST for these substances requires specific arrangements, such as: adding $Ca2^{+}$ to measure daptomycin susceptibilities; providing fresh medium and a low oxygen concentration to determine tigecycline susceptibilities; adding detergents to prevent surface attachment of lipoglycopeptides like dalbavancin; cefiderocol as a novel siderophore cephalosporine requires iron-depleted media for correct AST determination; and colistin AST is only reliable when performed by broth microdilution; just to mention a few of the manifold specificities and requirements. These technical peculiarities complicate an automation and quite often require manual handling, a good standardization and experienced personal to reliably determine susceptibilities against last resort antibiotics phenotypically. The mechanisms of last resort antibiotic resistances are often either multifactorial (daptomycin, carbapenems) or partly or completely unknown (tigecycline, cefiderocol, ceftaroline). Other resistances may be mutation-driven (various *loci*) and in combination also gene-based, such as linezolid resistance in Gram-positive cocci or colistin resistance in Gram-negative healthcare pathogens. Therapeutic failure may also result from phenomena of tolerance and persistence to antibiotics, which do not lead to changes in MIC values [18]. Also, the occurrence of small-colony variants or heteropopulations (e.g., heterogeneous VISA) precludes an accurate diagnostic stewardship in many situations, especially under prolonged treatments and in infections with high bacterial loads (e.g., endocarditis, osteomyelitis, etc.). This all complicates a proper genetic determination or genomic prediction of resistance or makes it impossible, for instance, in the case of a novel and unknown resistance that could only be determined phenotypically [19].

Recent achievements in antibiotic development introduced highly specific and specialized substances into clinical practice. For instance, novel beta-lactamase and carbapenemase inhibitors that complement beta-lactam and carbapenem therapy, but that require an adapted and targeted application. These inhibitors are highly active against some resistance enzymes but lack activity against others requiring a detailed and advanced diagnostic, where ideally the individual carbapenemase class should be determined in order to streamline and finetune antibiotic

treatment (e.g., vaborbactam is only active against KPC carbapenemases; avibactam is active against class A and D carbapenemases but not against metallo-β-lactamases of class B like NDM or VIM). A proper genetic or genomic diagnostic will ease and allow a targeted application of these highly effective compounds. In line with terms of an 'antibiotic stewardship', this novel diagnostic understanding gave birth to the term 'diagnostic stewardship' [20].

Resistance determination for surveillance purposes & screening

Results of classical, phenotypic AMR determination enter surveillance schemes and activities at the local, regional, national and international levels (e.g., EARS Net [21]). It is thus possible to recognize trends and developments, but the phenotypic assessment is purely descriptive and lacks information to explain causal links for most trends. As a simple example, rates of co-resistances in MRSA and VRE are declining. However, resistance properties to fluoroquinolones, gentamicin and macrolides do not get lost in those bugs; instead novel disseminating strain types, which contain a narrower spectrum of co-resistances, substitute the previously spread variants. In a background of some phenotypically assessible trends, the complexity of underlying mechanistic trends may be masked. Exemplarily, the increasing trend of VRE in Central Europe and Germany is driven by only a single genotype, *vanB*, disseminating more frequently, whereas rates of *vanA*-type resistance remained constant. In addition, the rapid increase in VRE is dominated by a few clonal types (ST117, ST80) and is not evenly distributed across the species *Enterococcus faecium*. Carbapenem resistance in *E. coli* and *Klebsiella* follows different trends over Europe [21]. However, only genomic analyses allow clonal and resistance trends with a sufficient analytical depth to understand underlying mechanisms and forces. In Northern America and Southern Europe, KPC-mediated carbapenem resistance in epidemic *Klebsiella pneumoniae* strains determine the high rates, whereas in Central Europe and Northern Africa, OXA-48-mediated carbapenem resistance dominates. Genome-based analyses depicted the role of horizontal gene transfer in spreading this resistance type in addition to clonal spread [22]. The detailed recognition of the emergence and rapid spread of novel resistance genes is only possible by genome-based surveillance, for instance, when identifying a novel OXA carbapenemase gene $bla_{\text{OXA-244}}$ spreading all over Europe [23]. In summary, to understand AMR trends, knowledge of the pathogens, their phylogenetic relatedness and the vehicles harboring the resistance determinants is essential to follow and understand AMR trends and follow their transmission routes.

Conclusion

Obviously, there is no 'one size fits all' method. Now and in the near future, phenotypic antibiotic susceptibility testing (AST) remains the gold standard and is often legally required for guiding patient therapy. Phenotypic and partly genotypic AST is internationally highly standardized; genome-based resistance prediction is technically highly advanced and reliable to a large extent, but mainly lacks harmonization and standardization. Genetic methods complement phenotypic AST by helping to confirm or falsify results. MDR (multidrug-resistant) pathogens extensively armed with last resort resistances require highly advanced AST methods for a 'diagnostic stewardship' like in the case of the novel carbapenemase inhibitors. Genomic AST prediction works well for well-known antibiotic classes with a defined genotype (single-nucleotide polymorphism gene presence), but time-to-result is too long to guide therapy (a few exceptions exist like mycobacteria). Many novel AMR phenotypes to last resort antibiotics may lack a distinct and clear genetic correlate; however, phenotypic AST may also be challenging here as well. A meaningful and reliable AMR surveillance requires genomic AST.

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