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The Poisoned Well: Enhancing the predictive value of antimicrobial susceptibility testing in the era of multidrug–resistance

Running Title: The Poisoned Well: Enhancing AST predictive value

Thea Brennan-Krohn[a,b], Kenneth P. Smith[a], and James E. Kirby[a#]

[a]Department of Pathology, Beth Israel Deaconess Medical Center

[b]Division of Infectious Diseases, Boston Children's Hospital

T. B-K. and K.P.S. contributed equally to this article.

#Corresponding Author

James E. Kirby

Beth Israel Deaconess Medical Center

330 Brookline Avenue - YA309

Boston, MA 02215

jekirby@bidmc.harvard.edu

Phone: 617-667-3648

Fax: 617-667-4533

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Abstract

Antimicrobial susceptibility testing (AST) is a fundamental mission of the clinical microbiology laboratory. Reference AST methods are based on bacterial growth in antibiotic doubling dilution series, which means that any error in the reference method is inherently at least two-fold. We describe the origins of current AST reference methodology, highlight the sources of AST variability, and propose ideas for improving AST predictive power.

Dilution-based antimicrobial susceptibility testing (AST) methods have been used to assess antimicrobial activity since the discovery of penicillin. In fact, Alexander Fleming himself used a tube-based dilution method for quantifying penicillin activity of different fungal culture filtrates (1) and even earlier had performed both diffusion- and dilution-based experiments to quantify the activity of lysozyme (2). Initially, performance of AST assays varied significantly in terms of media composition, inoculum size, incubation conditions, and antibiotic purity (3, 4). However, over the past several decades, AST has undergone a significant degree of procedural standardization.

Use of a variety of antimicrobial dilution series (e.g., sub-doubling dilutions) was described in early investigations of AST (5), but laboratories soon settled on a 2-fold dilution series. This geometric interval was chosen both for ease of performance and because of the observation that gradual, progressive inhibition around the MIC made determination of an exact MIC in finer dilution series challenging (4, 5). Inherently, however, any error in a doubling dilution series represents at minimum a two-fold difference, a point that was recognized as early as the 1940's (5).

The emergence of antibiotic resistance decreased the probability that an empiric antimicrobial regimen will be effective and thereby drove widespread implementation of AST in clinical laboratories. This practice was further expanded after establishment of correlations between *in vitro* susceptibility and clinical efficacy (6, 7). In the first decades of antibiotic use, the broth macrodilution method was commonplace for performing doubling dilution testing (3). However, as AST use increased, this cumbersome method was supplanted by a standardized broth microdilution assay. This miniaturization was facilitated by the introduction, in the 1960s,

of microtitration equipment that allowed for efficient, reproducible serial dilutions of antibiotics in 96-well plate format (8).

Early systematic evaluation of the broth microdilution method showed that 90-95% of MIC results were ±1 dilution from the median or mode for most antimicrobial/organism combinations (9). However, some clinical strains may exhibit even greater variability. For example, investigations in our laboratory have found that the percent of repeat broth microdilution MIC values that fall within ±1 dilution of the modal MIC ranges from 76% to 97% among different *Enterobacteriaceae* clinical isolates in a highly resistant strain set (unpublished data). For clinical isolates whose MICs fall near a susceptibility breakpoint, this variability results in categorical interpretive differences (that is, differences in classification of an isolate as susceptible, intermediate, or resistant) on repeat testing. This fact may be underappreciated by clinicians and laboratorians, and is not obvious in the absence of repeated testing, which is not generally performed in a clinical setting. The lower reproducibility for different types of clinical strains may not reflect the common experience with standard quality control strains (for example, *E. coli* ATCC 25922 or *S. aureus* ATCC 29213), which are specifically chosen for testing consistency and typically show ≥95% of values falling within ±1 dilution of a modal MIC (10-12).

To date, few studies have systematically evaluated the sources of AST variability, which likely has both biological and technical underpinnings. For example, biological variability may be introduced through use of different growth phases (13), inoculum densities, incubation conditions (e.g. duration, temperature, humidity, oxygen and carbon dioxide concentrations), or media (14). However, some proportion of biological variability is uncontrollable, as individual

organisms within clonal populations display phenotypic heterogeneity (15), likely related to stochastic epigenetic effects.

Significant progress has been made in reducing technical variation in AST through both procedure standardization and development of new technologies for panel preparation. Specifically, organizations such as the CLSI and EUCAST now provide guidance in terms of standards for media, incubation conditions, and assay performance (11). Furthermore, systematically quality-controlled broth microdilution panels prepared using automated liquid handling (rather than manual dilution) are now commercially available (16), minimizing, if set up properly, the cumulative error inherent in manual preparation of a two-fold dilution series.

However, some components of the AST process have proven more difficult to standardize. One procedure for which there is significant variability is the preparation of bacterial suspensions to match a 0.5 McFarland standard (17). Furthermore, 0.5 McFarland suspensions of organisms with different sizes, shapes, and clustering may yield colony forming unit counts that differ by several fold. This variability, reflected in the 4-fold range of acceptable colony forming unit inoculum outlined in CLSI guidelines (11), may hypothetically further contribute to MIC variability for antimicrobials that display an inoculum effect (18, 19). As such, improved, accessible methods of inoculum standardization and further investigation to elucidate the effect of inoculum density on MIC results for different organisms are needed.

The relative lack of MIC precision undoubtedly has clinical consequences. In addition to guiding treatment decisions on a per patient basis, AST and resultant MIC values are also used to investigate and define pharmacodynamic (PD) parameters that predict *in vivo* response to therapy. MIC breakpoints are established based on these PD studies, which correlate *in vitro*

97 organism susceptibility, achievable levels of antibiotic *in vivo,* and clinical outcomes.
98 Paradoxically, techniques for quantifying the levels of antimicrobials in blood and tissue are very
99 precise, with typical coefficients of variation ≤20% (20), while MIC assays, as mentioned
100 previously, may have 2-fold errors. Of note, an error of one 2-fold dilution represents a greater
101 absolute difference at higher antibiotic concentrations with the corresponding wider spacing of
102 dilutions. This intrinsic error represents a significant and well-recognized limiting factor in the
103 clinical applicability of PD analyses (21).

104 Therefore, more precise and accurate AST assays would provide several benefits. They
105 would improve PD modeling, support better clinical AST calls on individual patient isolates, and
106 allow "personalized" antimicrobial dosing. More specifically, as organisms develop significant
107 resistance and become effectively untreatable with available antimicrobials, salvage therapy
108 becomes a more pressing need. It has been recognized that, for some antimicrobials, dose or
109 dosing frequency may be increased while skirting the abyss of unacceptable toxicity. This
110 concept has been codified in the new susceptible dose-dependent criteria recently promulgated
111 by the CLSI for the drug cefepime (22). Here, alternative dosing regimens are proposed to treat
112 organisms with elevated MICs (4 or 8 μg ml^{-1}) that might otherwise not be considered treatable
113 and which are in fact considered resistant at an MIC of 8 μg ml^{-1} by current EUCAST criteria
114 (http://www.eucast.org/clinical_breakpoints/). Importantly, the trade-offs between potential for
115 enhanced therapeutic effect and increased risk of toxicity might only be acceptable if we are
116 confident that the MICs measured are accurate and reflect true potential for cure. Such critical
117 assessments are of particular importance for drugs with narrow safety margins such as
118 aminoglycosides and colistin (23, 24).

One approach to improving accuracy of MIC determinations is to use a dilution series with finer than two-fold dilution intervals. The availability of automated liquid handlers and other programmable antibiotic dispensing systems means that the previously time-consuming and error-prone process of preparing sub-doubling dilutions is no longer a true impediment. Finer dilutions could be discontinuous and concentrate around critical decision points, such as cutoffs bordering safety margins and breakpoints, and include finer gradations bracketing quality control strain ranges to allow greater sensitivity to detect subtle drift in panel performance (25, 26).

It is also possible that the standard MIC is not the ideal measure for predicting response to therapy for individual patients or for PD modeling. Although the current AST reference standard is visual inspection for complete inhibition of bacterial growth, it is clear that many antimicrobials exert effects below the MIC that cannot be quantified by eye. Correspondingly, substantial therapeutic effect is often observed even for organisms that are categorized as resistant by standard MIC measurements (27). To gain more information regarding sub-MIC-based inhibitory effects of antibiotics and support further exploration of the relevance of these effects during therapy, bacterial growth inhibition can be modeled as a dose-response curve using spectrophotometric measurements (28) to yield MIC, IC_{50} (concentration required to reduce final cell absorbance by 50%), and Hill slope parameters (28, 29). In addition, advances in automated testing may permit repeated MIC measurements in a clinically actionable time frame, thereby allowing for the detection of strains with inconsistent susceptibility profiles. Another potentially informative variable is the dimension of time. Growth kinetic assessments are already used in clinical systems such as Vitek2 (Biomérieux, Durham, NC) to extrapolate

MICs from a limited number of antimicrobial concentrations (30). However, the full potential of kinetic measurements in predictive AST determination is likely underexplored. Lastly, newer techniques for real-time assessment of bacterial viability, in addition to bacterial growth inhibition assessed by standard testing, may provide additional prognostic value. Ultimately, a multi-parameter analysis including several or all of these measures may provide the most informative readout.

With the development of new technologies such as automated liquid handling and the adoption in clinical settings of algorithms that can incorporate numerous components of a multidimensional readout, we expect the predictive capabilities of AST will be improved significantly in the future. Clearly much research and dedicated work lies ahead. However, the antimicrobial resistance threat is looming, and it is a challenge that we must embrace.

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