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COMMENTARY

The Poisoned Well: Enhancing the Predictive Value of Antimicrobial Susceptibility Testing in the Era of Multidrug Resistance

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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 inherently represents at least a 2-fold difference. We describe the origins of current AST reference methodology, highlight the sources of AST variability, and propose ideas for improving AST predictive power.

KEYWORDS antimicrobial activity, minimal inhibitory concentration, precision, susceptibility testing

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 composition of media, 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., subdoubling 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 2-fold difference, a point that was recognized as early as the 1940s (5).

The emergence of antibiotic resistance decreased the probability that an empirical 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 efficient, reproducible serial dilutions of antibiotics in 96-well plate format (8).

Early systematic evaluation of the broth microdilution method showed that 90% to 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 proportion of repeat broth microdilution MIC values that fall within ±1 dilution of the modal MIC

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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. Clinical AST methods that are currently in use are too time-consuming to allow routine repeat testing of isolates, but if new automated methods sufficiently increase the speed and efficiency of AST, such replicate testing may become practical and may be applied in a targeted fashion to isolates and antimicrobials which, based on underlying resistance patterns, may be at increased risk for variable AST results. If such replicate testing were to be performed, it would be possible to report a result such as the mode of replicate MIC assays. Furthermore, the finding that a particular strain shows greater-than-expected variability on replicate assays for a given antibiotic could be included in a report to alert clinicians of potential decreased predictability in antibiotic responses. The lower reproducibility for different types of clinical strains may not reflect the common experience with standard quality control strains (for example, *Escherichia coli* ATCC 25922 or *Staphylococcus aureus* ATCC 29213), which are specifically chosen for testing consistency and typically show $\geq$95% of values falling within $\pm$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, and 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 2-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 characteristics may yield CFU counts that differ by severalfold. This variability, reflected in the 4-fold range of acceptable CFU inocula outlined in CLSI guidelines (11), may hypothetically further contribute to MIC variability for antimicrobials that display an inoculum effect (18, 19). As such, there is a need for improved, accessible methods of inoculum standardization, such as automated spectrophotometric analysis and inoculum preparation, as well as for further investigation to elucidate the effect of inoculum density on MIC results for different organisms and the potential application of differing inoculum density standards for different species.

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* organism susceptibility, achievable levels of antibiotic *in vivo*, and clinical outcomes. Paradoxically, techniques for quantifying the levels of antimicrobials in blood and tissue are very precise, with typical coefficients of variation being $\leq$20% (20), while MIC assays, as mentioned previously, may have 2-fold errors. Of note, an error of one 2-fold dilution represents a greater absolute difference at higher

antibiotic concentrations with the correspondingly wider spacing of dilutions. This intrinsic error represents a significant and well-recognized limiting factor in the clinical applicability of PD analyses (21).

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

One approach to improving accuracy of MIC determinations is to use a dilution series with dilution intervals that are finer than 2-fold. The availability of automated liquid handlers and other programmable antibiotic dispensing systems means that the previously time-consuming and error-prone process of preparing subdoubling 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 could include finer gradations bracketing quality control strain ranges to allow greater sensitivity to detect subtle drift in panel performance (25, 26). While decreasing dilution intervals will not reduce the biological variability that may play a role in inconsistent MIC results, it will provide increased data about the true location of the MIC within the dilution series. Initially, subdoubling dilution results would likely be rounded up to the nearest doubling dilution to allow interpretation by standard susceptibility criteria. In time, however, the widespread adoption of finer dilution series could facilitate development of new, more precise breakpoints that would not be required to fall on doubling dilutions.

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 to 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, the IC_{50} (the concentration required to reduce final cell absorbance by 50%), and Hill slope parameters (28, 29). The data on sub-MIC effects provided by the IC_{50}, for example, are expected to provide a more robust measure of antibiotic effect than a single MIC value (29), although standardized interpretive criteria like those used for evaluation of MICs would need to be developed before the IC_{50} could be used in the clinical laboratory. Future *in vivo* and clinical studies could determine whether dose-response parameters such as IC_{50} and Hill slope have implications for therapeutic parameters such as antibiotic dosing. 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 underex-

plored. 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 multiparameter analysis that includes 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 that the predictive capabilities of AST will be improved significantly in the future. Clearly, much research and dedicated work lie ahead. However, the antimicrobial resistance threat is looming, and it is a challenge that we must embrace.

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