Updating Antimicrobial Susceptibility Testing Present and Future Relevance

CYNTHIA B. SCHOFIELD

LEARNING OBJECTIVES:

1. Discuss the "state of the art" of antimicrobial susceptibility testing (AST) from the National Laboratory System (NLS) surveys.
2. Summarize the critical factors in disk diffusion and broth dilution testing.
3. Explain the potential sources of error inherent in AST methods.
4. Discuss breakpoints and reasons for microbiologists concern.
5. Discuss the controversy surrounding AST interpretive values.
6. Compare and contrast AST and molecular assays for routine laboratory use.

INDEX TERMS: *Enterococcus* species, methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pneumoniae*, *Klebsiella pneumoniae*

ABBREVIATIONS: AST-antimicrobial susceptibility testing; NLS-National Laboratory System; CDC-Center for Disease Control and Prevention; CLSI-Clinical and Laboratory Standards Institute; MIC-minimal inhibitory concentration; MH-Mueller-Hinton; CAP-College of American Pathologists; PK/PD-pharmacokinetic/pharmacodynamics; UTI-urinary tract infection; CSF-cerebrospinal fluid; FDA-Food and Drug Administration; EUCAST-European Committee on Antimicrobial Susceptibility Testing; AUC-area under the concentration curve; PCR-polymerase chain reaction; HAI-hospital-associated infection; CAI-community-associated infection; MRSA-methicillin-resistant *Staphylococcus aureus*; VRE-vancomycin-resistant *Enterococcus* sp.; MTB-*Mycobacterium tuberculosis*; ESBL-extended-spectrum beta lactamase; KPC-*Klebsiella pneumoniae* carbapenem-resistant; MDR-multi-drug resistant.

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Cynthia B. Schofield, MPH, MT (CAMT), VA San Diego Healthcare System (retired), San Diego, CA

***Address for Correspondence:** Cynthia B. Schofield, MPH, MT (CAMT), VA San Diego Healthcare System (retired), 7050 Weller St., San Diego, CA, (858) 450-9651, cschofield@san.rr.com*

State of the Art

Testing methods and reporting of results vary widely among clinical, reference and public health laboratories. Accordingly, institutional size and resources dictate both antimicrobial susceptibility testing (AST) needs and the cost effectiveness of test methods. Results of evaluations from 102 laboratories by the National Laboratory System (NLS) between 2001 and 2005 proved fewer than 50% of these laboratories published antibiograms of local resistance patterns and fewer than 50% adhered to the guidelines and standards established by the Clinical and Laboratory Standards Institute (CLSI).

The effort to improve performance and adherence to CLSI standards and to assure rapid detection and reporting of antimicrobial resistance was undertaken by the NLS. NLS consists a group of microbiologists from the University of Washington, the University of California at Los Angeles and the Centers for Disease Control and Prevention (CDC).

The NLS investigators surveyed the labs in 2001 and 2005 to determine the effect of 2003 and 2004 interventions. The interventions included AST workshops according to CLSI guidelines. Investigators trained local faculty in the use of a CDC AST self study and distributed free CLSI documents. Web sites and teleconferences were instituted as educational supplements. The 2001 assessment compared case studies of AST performance when testing *Enterococcus* sp., methicillin-resistant *S. aureus* (MRSA) and *S. pneumoniae* among the 102 laboratories. In a 2002 survey, changes in case studies from the previous year

were noted and recorded.

Changes over a period of 5 years were tabulated by the surveys (2001 to 2005) in the 102 laboratories of small, moderate and large hospitals, physician office/clinics, as well as reference and public health laboratories in the state of Washington. Correct responses for the 3 organisms tested by case study showed improvement in all laboratories from 2001 to 2005. Although the study was small, there was an impressive change in small hospital (< 100 beds) laboratories where AST accuracy for *Enterococcus* sp. and *S. pneumoniae* increased by > 30%. As a result of NLS programs, the percentage of laboratories performing AST and following CLSI standards increased by 20% from 2001 to 2005.[1]

Critical Factors

The following examples illustrate the importance of CLSI standards for AST.

As commonly performed in small to moderate size hospitals, the Kirby-Bauer qualitative method of disk diffusion is designed to test only rapidly-growing organisms with CLSI interpretive criteria available. Thus, fastidious bacteria, e.g. *Campylobacter* spp. and anaerobic bacteria that require special media and procedures often are tested only in reference laboratories. To avoid delays caused by referral larger hospitals, medical centers, reference and public health laboratories, where cost is a lesser consideration, usually perform the quantitative methods (with MICs), broth (or agar) dilution testing either manually or by automated system. Standardized by CLSI, the media used in the above methods requires precise pH and cation content, as well as specific concentrations of calcium and magnesium ions. Detection of oxacillin resistance in staphylococci requires further addition of specific amounts of sodium chloride in both agar and broth dilution methods. Mueller-Hinton (MH) agar is poured to a depth of 3-4 mm for disk diffusion. All system component instructions—antimicrobial agent disks, agar plates and antimicrobial tray storage requirements—must be followed precisely. Many of these items are first on the College of American Pathologists (CAP) inspector's checklist (www.accred@cap.org, www.cap.org).

Inherent in the procedure steps are potential sources of error, for example: inoculum purity, inoculum dilution density, incubation temperature and time. For example, incubation is 16-20 hours with broth dilution and 16-18 hours with disk diffusion in ambient air or room temperature. Incubation times must be extended for staphylococci when testing oxacillin and vancomycin MICs. For staphylococci and enterococci when testing vancomycin, a full 24 hours is required for the expression of inducible genes such as *mec*A, *van*A and *van*B.

Endpoint interpretation, whether the method is manual, semi-manual or automated, requires periodic monitoring and calibration. Reference organism strains are tested according to quality control protocol with all deficiencies thoroughly investigated, documented, and followed by corrective action.

Reporting errors may occur due to lack of correlation of *in vitro* with *in vivo* activity, e.g. narrow and expanded spectrum cephalosporins and aminoglycosides tested against *Salmonella* spp. and *Shigella* spp. For organisms in this category antibiotic results are not reliable and should not be reported.[2]

Controversial Factors

AST results are based on breakpoints (interpretive criteria), which are the values that determine the categories susceptible, intermediate and resistant. Established by CLSI, breakpoints incorporate the following parameters: MICs and disk diffusion zone diameter distributions based on surveys of recent clinical isolates, drug stability, pharmacokinetic/pharmacodynamic (PK/PD) efficacy correlation, relation of testing results and outcome statistics and the impact of resistance mechanisms on testing results.

An example of the confusion and concern of physicians regarding breakpoints was illustrated when CLSI (2010) changed the cefazolin breakpoint for susceptibility from 8μg/mL to 1μg/mL based on the 1g/8hr drug manufacturer dosage recommendation. When complaints that urinary isolates tested at the intermediate level led to alternative prescribing of carbapenem antibiotics, CLSI determined to accommodate the highest recommended dose of 2g/8hr, again changing the cefazolin breakpoint to ≤ 2 μg/mL and read as susceptible. Although the new breakpoint was based on the maximum dosage for cefazolin, CLSI did not recommend including this information in the

laboratory's report.[3]

A committee of clinical microbiologists and industry representatives pointed to the draw-backs when breakpoints are used, while suggesting a new approach to interpreting AST results. Use of personalized antibiotic reports would allow for variation in the site/location of infection (UTI, CSF, blood, etc.), drug administration path (oral versus intravenous) and dosage (high versus low). Specifically, antibiotic reports (antibiograms) are not tailored to infections in the blood, rather to those of urine, wound, tissue, etc.[3,4]

Inconsistency among the organizations that determine the criteria for setting breakpoints is another concern of clinical microbiologists. In the United States, the Food and Drug Administration (FDA) and the CLSI and in Europe, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) have the task of determining the MIC breakpoints. Despite use of similar frequency distribution analysis, assessment related to known resistance mechanisms, and evaluation based on drug levels (i.e. PK/PD analysis, response rates/clinical correlation) the breakpoints may differ.

PK information includes serum peak and trough concentrations of proposed antibiotic drug regimens graphed over time following the criteria of bioavailability, volume of distribution, metabolism, excretion/clearance and half-life of the antibiotic. PD analyses involve the time interval that serum levels exceed the drug MIC, peak ratios of serum level to MIC and ratios of area under the serum concentration-time curve (AUC) to MIC. The confounding *in vivo* factors involved in a patient's response to therapy (e.g. immune response, weight, allergies, etc.) make evaluation of clinical correlation data derived from AST extremely difficult. *In vitro* AST results using CLSI methods attempt to correlate with therapeutic outcomes by becoming part of the breakpoint determination.

Although data from single-microbe infections and polymicrobial infections are evaluated separately, the variability inherent in clinical outcome data makes establishment of clear breakpoints relatively unlikely. Individual immune response patterns combined with the effects of multiple antibiotic therapy make clinical correlation difficult if not impossible to assess. Improved guidance, supervision and monitoring in clinical laboratories are necessary if understanding and implementation of CLSI modifications are to be effective. The consensus of another group of discussants was that advancement of molecular methods to provide rapid detection of resistant determinants had the potential of making AST irrelevant in the future.[3,4]

The clinical predictive value of *in vitro* AST may not accurately reflect a favorable versus unfavorable patient outcome. Factors such as bacterial virulence, variation in the response of a patient's immune system, polymicrobical infections requiring multiple antibiotic therapy all make predictive determination unclear. The "90-60" Rex and Pfaller (2002) rule as applied to studies of infection with a single bacterium treated with a single antibiotic follows: in 90-95% of susceptible cases the predictive value holds; however, *in vitro* tests predict resistance or failure in < 35% of patient cases depending on a diversity of host response factors.[5]

PCR assays have replaced AST for detection of certain resistance determinants, particularly for those known to cause HAI and CAI, e.g. *mec*A in *S. aureus* and extended-spectrum beta lactamase (ESBL) enzymes in *E. coli*, *K. pneumoniae*, and other species of Enterobacteriaceae. The greater concern with this approach is that detection of the causes of polymicrobial infection requiring PCR multiplex assays are more complicated to develop and may lack the sensitivity of single primer PCRs. The on-going challenge of molecular testing to assure gene expression rather than gene carriage continues unabated.[5,6]

To alleviate the controversy surrounding the reporting of results, a group of microbiologists and industry representatives discussing the problems of AST testing and reporting suggested a more objective electronic approach. Electronic software would be developed to incorporate intrinsic resistance, susceptibility testing and optimal antibiotic guideline information, personalized per patient, together with programs that report the therapeutic antibiotic concentration at a specific body site. These programs would flag the physician or clinical pharmacist when testing revealed the patient was not receiving optimal treatment. Personalized reporting was suggested as the model to replace the present cascade reporting.

In the former method, a software program determines

the appropriate antibiotic based on an individual patient's condition (e.g. immune status, weight, allergies, creatinine clearance and albumin levels), the organism and MIC, as well as drug interactions and cost. Cascade reporting allows placement of antibiotics in specific drug classes, first based on the cost of the drug and second on its susceptibility results, while the final report displays the least expensive drug testing as susceptible. For example, presence of the AAC6' resistant gene that hydrolyzes tobramycin and amikacin, but usually not gentamicin, would produce a report of the less expensive gentamicin as susceptible, while suppressing results for the resistant and more expensive tobramycin and amikacin. Without guidelines attesting to the inappropriate *in vivo* use of aminoglycosides when AAC6' is present, a drug with potential adverse effects may be prescribed.

There are no published guidelines for reporting to aid laboratories when unusual patterns of resistant determinants occur. Some but not all drugs in a certain class may test as susceptible and those that seem appropriate from *in vitro* testing may not be advisable for *in vivo* use.[4]

Despite setbacks dating ten years from the first PCR assay for methicillin-resistant *Staphylococcus aureus* (MRSA), direct molecular detection of *mec*A as an alternative to AST, now may be mandated by its inherent speed and sensitivity. However, problems that continue to thwart this approach include rare false positive results, undetected mutations, lack of clinical specificity, and the inability to detect resistance determinants in gram-negative bacilli. More than 200 genotypes and unique ESBLs have been identified, all with distinct phenotypic variations. For example, TEM10 differs by a single amino acid from TEM12, but for strains that carry these enzymes, the MICs of ceftazidime differ by >100-fold. Understandably the focus has concentrated on screening for gram-positive organisms, e.g. MRSA, VRE, rifampin-resistant MTB in an attempt to prevent hospital-associated infection.[7]

Diagnosis of resistant gram-negative organisms in HAI and CAI has become problematic with the increasing number of resistance determinants that require direct detection from clinical material for confirmation. Detection of enzymes such as the KPC carbapenemases found in Enterobacteriaceae other than *K. pneumoniae* and the metallo-beta lactamases (zinc-dependent carbapenemases) require multiplex assays to screen the maximum number of determinants, e.g. NDM-1, $bla_{IMP,}$ bla_{VIM}, and $bla_{OXA..}$ Current technology is limited by multiple manipulations, slower turn-around-time and the inability to detect carriage versus gene expression. Yet, the possibility of combining AST methods and molecular detection of resistance determinants holds promise for the future to replace empiric therapy and eliminate the sometimes greater than 48 hour AST delay.[7]

In the article Challenging Cases, questions arose to suggest that molecular detection of resistant determinants may have had a more positive effect on patient outcome. The following examples are summarized here.

Acinetobacter baumannii

Growth of *A. baumannii* from a patients' blood cultures proved resistant to amikacin, ampicillin-sulbactam, ceftazidime, ceftriaxone, ciprofloxacin, gentamicin, imipenem, piperacillin/tazobactam, tobramycin and trimethoprim-sulfamethoxazole. Wound cultures were resistant to all but tobramycin and amikacin, which was intermediate. Both patients died of necrotizing fasciitis caused by the same MDR organism despite extensive surgical debridement. Would detection with multiplex PCR assay have been successful in these cases? It is unlikely that early intervention and treatment with wound and abdominal fluid susceptible ampicillin-sulbactam would have reversed the toxic effects of *A. baumannii* in these patients.[8]

Escherichia coli ST131

Failed empiric therapy with fluoroquinolones was cited as the cause of one sister's demise. A second treatment with piperacillin/tazobactam based on an antibiogram was also unsuccessful. Susceptibility testing results showed the isolate was resistant to fluoroquinolones and ESBL-producing cephalosporins but susceptible to piperacillin/tazobactam, amikacin, carbapenems and trimethoprim-sulfamethoxazole. However, the belated change to meropenem failed and the patient died. Isolates from both sisters exhibited $bla_{CTX\text{-}M\text{-}15}$, which encodes the *E.coli* ST131 strain associated CTX-M-15 ESBL variant. Would direct gene/enzyme detection have prevented the failed therapy and fatal outcome in this case?[9]

Conclusion

Many questions remain unanswered regarding the ideal method of testing the numerous resistant organisms lurking in both hospital and community facilities. Reliance on both empiric therapy as well as the use of standardized AST will undoubtedly predominate in the United States into the next decade. However, the influx of successful molecular techniques promises faster, more accurate identification/detection to improve therapy and patient outcome. At least two of the patient cases presented here may have experienced a positive outcome if molecular testing had been applied.

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