

## Evaluation of the Performance Parameters of a Prediluted, Quantitative Antibiotic Susceptibility Test Device

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A multiwelled plastic strip containing prediluted antibiotics was tested. Antibiotics available at the time of testing included ampicillin, penicillin, methicillin, erythromycin, clindamycin, tetracycline, and minocycline. The effects of inoculum size, inoculum volume, temperature, and time of incubation were determined. A limited clinical evaluation using laboratory strains of selected bacteria proved this product to be a rapid, economical, and reliable method for the determination of the minimal inhibitory concentration of antibiotics.

There is a continuing need for quantitative antibiotic susceptibility methods that do not require the purchase of expensive equipment or hours of a technologist's time for processing.

The antibiotic susceptibility test (AST; Lederle Laboratories, Pearl River, N. Y.) device is a multiwelled plastic strip containing prediluted concentrations of an antibiotic. Inoculation and incubation of these strips results in the determination of the minimum inhibitory concentration (MIC) of the antibiotic to be tested. The MIC of antibiotics may be determined by a number of methods, including agar dilution (2), broth dilution (6), and microdilution (7, 8). No universally recognized reference methods exist for these procedures, although investigators have examined variables and proposed procedures. The present joint study evaluates the performance parameters of the AST strip.

### MATERIALS AND METHODS

**Antibiotics.** The AST is an injection-molded polystyrene multiwelled chamber (Fig. 1). The 10 individual wells hold a maximum fluid volume of 400  $\mu$ l and are covered by a removable snap-type flexible plastic cover. Each strip contains serial decreasing amounts of an antibiotic in a dried state. Well no. 1 contains 12.5  $\mu$ g of antibiotic, well no. 2 contains 6.25  $\mu$ g, etc. Well no. 10 contains no antibiotic. Antibiotics available at the time of testing included ampicillin, penicillin, methicillin, erythromycin, clindamycin, tetracycline, and minocycline. (Information from Lederle Laboratories indicated that these antibiotics were stable at room temperature for a minimum of 6 months.) The strips were stored at room temperature in their packages prior to use. Antibiotics used in the microdilution and agar dilution procedures were obtained as USP reference powders and stored at  $-20^{\circ}\text{C}$  until used.

**Control organisms.** The following reference strains for the stated antibiotics were employed in

the evaluation of test conditions for the AST: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Sarcina lutea* ATCC 9341, *E. coli* WHO-5 (ampicillin, clindamycin); *S. aureus* WHO-4 (penicillin); *S. aureus* 2834 (methicillin); *S. aureus* WHO-6 (erythromycin) and *E. coli* WHO-16 (tetracycline, minocycline).

The limited clinical phase of the study evaluated bacterial strains routinely isolated from the clinical microbiology laboratories of the University of Connecticut Health Center and the Long Island Jewish-Hillside Medical Center. All cultures were either tested immediately after isolation or maintained on Trypticase soy agar slants. *S. aureus* 25923 and *E. coli* 25922 served as the quality control organisms included with each series of tests performed.

**AST procedure.** An AST strip was removed from its package, unwrapped, and placed on a level table. The cover was removed, and 200  $\mu$ l of inoculated growth medium was added by pipette to each of the nine antibiotic-containing wells. These wells contained an absolute amount of antibiotic. The volume of the inoculum, therefore, affected the actual MIC value. Thus, the use of 0.2 ml as inoculum approached the usual  $\log_2$  dilution of the antibiotics most closely. Two hundred microliters of inoculum was added to well no. 10, the growth control. The cover was replaced, and the strip was incubated at  $35^{\circ}\text{C}$  for the stated time.

**Disk diffusion susceptibility test.** The standardized disk test was performed in exact accordance with the method of Bauer et al. (1). All diffusion tests were accompanied by plates inoculated with *E. coli* ATCC 25922 and *S. aureus* ATCC 25923.

**Agar dilution susceptibility test.** The procedure was performed according to the recommendations of Ericsson and Sherris (2).

**Microdilution antibiotic susceptibility test.** The method of Tilton et al. (8) was followed.

**Determination of optimal inoculum concentration for the AST test.** Inocula of reference cultures in Mueller-Hinton (M-H) broth for each of the antibiotics to be tested were adjusted by nephelometry (Tilton et al. [8]) to  $10^4$ ,  $10^5$ ,  $10^6$ , and  $10^7$  colony-

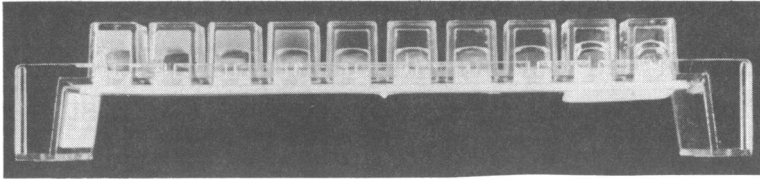


Fig. 1. The AST, an injection-molded polystyrene device for determining the MIC of antibiotics.

forming units (CFU) per ml. The AST strips were inoculated in quadruplicate, and incubated for 18 h at 35°C, and the MICs were determined as a function of each inoculum concentration.

**Effects of growth on the AST test.** Quadruplicate AST strips were inoculated with the reference strains at a concentration of  $10^5$  CFU per ml in four nutrient media: Trypticase soy broth (Difco), Eugon broth (Difco), M-H broth (BBL), and brain heart infusion broth (Difco). The strips were incubated at 35°C for 18 h and read.

**Effects of inoculum volume on the AST test.** AST strips were inoculated in quadruplicate with 200, 300, and 400  $\mu$ l of a  $10^5$  bacterial inoculum. The strips were incubated for 18 h and the MICs were determined.

**Time of incubation.** AST strips were inoculated with 200  $\mu$ l of a  $10^5$ -CFU/ml inoculum and incubated at 35°C. Strips were removed at 2, 4, 6, 8, 18, 24, 40, and 48 h. The MICs on two strips were determined visually, and the MICs of the other two strips were read by the addition of 50  $\mu$ l of iodinitrotetrazolium to each well. If growth was apparent, the tetrazolium was reduced to a red, insoluble formazan compound.

**Limited clinical evaluation.** Representative isolates from the clinical laboratories as well as control strains of *E. coli* 25922, *S. aureus* 25923, and *S. lutea* were tested by AST, disk diffusion, agar dilution, and microdilution.

The AST protocol was as follows: inoculum concentration,  $10^5$  CFU/ml; inoculum size, 200  $\mu$ l per well; growth medium, M-H broth; incubation temperature, 35°C; incubation time, 6 h; reading of end point, visually in reflected light.

**Limited clinical evaluation of fastidious organisms.** Selected isolates of *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Fusobacterium* sp., and *Bacteroides fragilis* were tested with ampicillin, clindamycin, tetracycline, and penicillin. The M-H medium for growth of the *Haemophilus* and *Neisseria* isolates was supplemented with either supplement C (Difco), yeast extract (1%) and hemoglobin (1%), or 10% Fildes reagent (Difco). The anaerobic gram-negative rods were incubated in a GasPak (BBL) for 24 h prior to reading. All inocula were standardized to  $10^5$  CFU per ml. The reference method employed was macrotube broth dilution (6) using identical media and environmental conditions.

## RESULTS

The first phase of evaluation of the AST focused on the establishment of test conditions.

The volume of inoculum added to the wells did not alter the MIC when the concentration of antibiotic per milliliter was adjusted for dilution. In most cases, the MICs were similar regardless of volume and no greater than a 1-dilution interval (d.i.) difference was observed. It was noted, however, that addition of 400  $\mu$ l of inoculum to a well caused overflow and resultant contamination of the plate. An inoculum of 200  $\mu$ l was employed for subsequent tests.

Three brands of manual pipettors with disposable tips were used to add a 200- $\mu$ l inoculum to the wells: Oxford, Clay-Adams, and Pierce (Pierce Chemical Co.). Thirty repetitive tests on each pipette with a methicillin AST strip and *S. aureus* 2834 indicated no change in MIC as a function of pipette type. The Clay-Adams pipettor was used for the remainder of the study because of rapid uptake and release of the inoculum and ease of operation.

Figure 2 shows the effect of inoculum concentration on the AST MICs. Penicillin demonstrated a significant inoculum effect due to the production of penicillinase by *S. aureus*. Inoculum concentrations of  $10^6$  CFU or greater per ml increased the apparent resistance of the test organism to penicillin. A similar effect was observed when *E. coli* was tested with clindamycin. The lack of inoculum effect with ampicillin, and the presence of one with clindamycin, is unexplained. Inocula of  $10^6$  to  $10^7$  CFU per ml caused sufficient turbidity in the medium so as to make visual reading of the MIC difficult. Lesser inocula resulted in extended periods of inoculation.

Figure 3 shows the effect of the growth medium on AST MICs. No marked differences in MIC were seen as a function of nutrient medium except that microorganisms appeared to be more susceptible to some antibiotics when growing in M-H broth. M-H broth was chosen for the remainder of the study for the sake of uniformity with other susceptibility test methods.

Experiments indicated that the AST could be used as a 1-day test of quantitative antimicrobial susceptibility. MICs of all antibiotics for the control cultures, *S. aureus* and *E. coli*, could be determined after incubation for 6 h. Differences of greater than 1 d.i. were not ob-

served when the strips were read at intervals of 6 to 48 h. Occasionally, extended incubation (12 to 18 h) was necessary when testing enterococci and some pseudomonads. It is critical that the strips be read in reflected light through the transparent side of the wells. This procedure allows the reader to shake the bacterial suspension in the well and observe growth. When the

test was read by looking down through the top of the well, turbidity changes could not be observed in 6 h. One of the authors (R.C.T.) added tetrazolium to the wells at 6 h to facilitate the reading of the MIC end point. Results after tetrazolium addition were identical to those in which turbidity alone was assessed. The time necessary to generate a reliable MIC was not shortened by a 2-h preincubation of the inoculum.

Table 1 shows 10 replicate MIC determinations for each of the antibiotics listed. In no case was there a replicate MIC greater than 1 d.i. from the mean of the 10 determinations. MICs, expressed as well numbers, of erythromycin, tetracycline, and minocycline were identical for the 10 replicates.

Table 2 summarizes comparative data between agar dilution, microdilution, broth dilution, disk diffusion, and the AST method using reference strains and the parameters determined to be optimal. In most instances, there was excellent agreement with the macrotube dilution result. In no instance was there interpretative disagreement between the disk diffusion test and any one or more of the dilution tests.

Table 3 is a compilation of data on the limited clinical evaluation of the AST. Gram-negative rods, exclusive of *H. influenzae*, *Fusobacterium* sp., and *B. fragilis*, were tested with ampicillin, tetracycline, and minocycline. Gram-positive bacteria were tested with ampicillin, clindamycin, erythromycin, methicillin, and penicillin. In some instances, isolates were tested by six methods: agar dilution, microdilution, broth dilution, disk diffusion, Autobac, and AST. Other isolates were tested only by AST and broth dilution. The presentation in Table 3 assumes that there is no significant discrepancy in results when there was a 1 d.i. or less difference in MIC as a function of method. Of five strains of *Streptococcus pneumoniae* tested, one isolate showed >1 d.i. difference when tested with penicillin. With either TD or AST, however, the isolate would

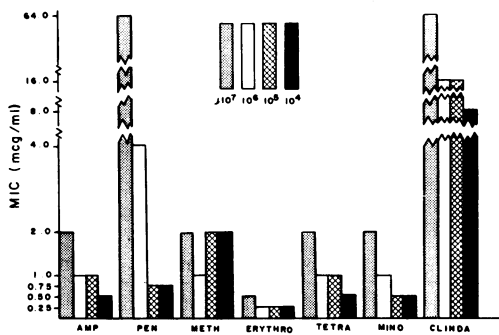


FIG. 2. Effect of inoculum concentration on AST MICs. Control organisms: *E. coli* WHO-5 (ampicillin, clindamycin); *S. aureus* WHO-4 (penicillin); *S. aureus* 2834 (methicillin); *S. aureus* WHO-6 (erythromycin); *E. coli* WHO-16 (tetracycline, minocycline).

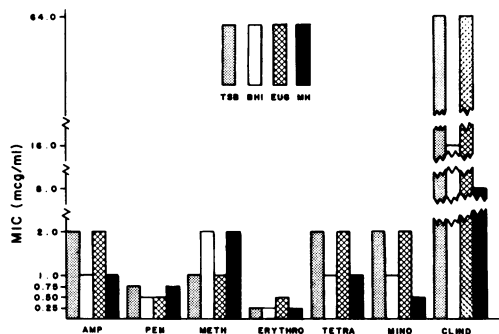


FIG. 3. Effect of growth medium on AST MICs. Control organisms: *E. coli* WHO-5 (ampicillin, clindamycin); *S. aureus* WHO-4 (penicillin); *S. aureus* 2834 (methicillin); *S. aureus* WHO-6 (erythromycin); *E. coli* WHO-16 (tetracycline, minocycline).

TABLE 1. Variation in replicate AST MICs

Antibiotic	Control organism <sup>a</sup>	Mean MIC	Range <sup>b</sup>	Standard deviation
Ampicillin	<i>E. coli</i> WHO-5	0.85	0.5 (3)-1.0 (7)	0.23
Penicillin	<i>S. aureus</i> WHO-4	0.48	0.25 (1)-0.5 (9)	0.07
Methicillin	<i>S. aureus</i> 2834	1.70	1.0 (3)-2.0 (7)	0.45
Erythromycin	<i>S. aureus</i> WHO-6	0.25	0.25 (10)	0.00
Tetracycline	<i>E. coli</i> WHO-16	1.0	1.0 (10)	0.00
Minocycline	<i>E. coli</i> WHO-16	1.0	1.0 (10)	0.00
Clindamycin	<i>E. coli</i> WHO-5	17.6	16.0 (9)-32 (1)	4.2

<sup>a</sup> Inoculum, 10<sup>5</sup> CFU per ml. Incubation was for 6 h at 35°C.

<sup>b</sup> Parentheses indicate number of replicates with the designated MIC end point.

TABLE 2. Comparison of AST, broth dilution, agar dilution, microdilution, and agar diffusion methods for antimicrobial susceptibility

Quality control strain	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>					
	Amp	Pen	Meth	Eryth	Clind	Tetra
<i>S. aureus</i> 25923						
AST	<0.25	<0.25	1.0	<0.25	<0.25	0.50
Broth dilution	<0.25	<0.25	1.0	0.5	0.50	0.25
Agar dilution	<0.25	<0.25	0.50	<0.25	<0.25	<0.25
Microdilution	<0.25	<0.25	0.50	<0.25	<0.25	<0.25
Disk diffusion	40 mm	42 mm	36 mm	25 mm	22 mm	29 mm
<i>E. coli</i> 25922						
AST	8	>64	>64	>64	>64	4
Broth dilution	4	>64	>64	>64	>64	4
Agar dilution	4	32	>64	>64	>64	2
Microdilution	4	32	>64	>64	>64	2
Disk diffusion	16 mm	6 mm	6 mm	16 mm	6 mm	20 mm

<sup>a</sup> Amp, Ampicillin; pen, penicillin; meth, methicillin; eryth, erythromycin; clind, clindamycin; tetra, tetracycline.

TABLE 3. Summary of isolates tested and discrepancies in AST results observed in a limited clinical trial

Organism	No. of isolates	Method <sup>a</sup>	Discrepancies <sup>b</sup>
<i>Staphylococcus aureus</i>	15	AD, MD, TD, AST, DD, AB	2 (Amp, MD); 2 (Meth, MD); 1 (Meth, AD); 1 (Pen, AD)
<i>Klebsiella pneumoniae</i>	15	Same	1 (Tetra, DD)
<i>Escherichia coli</i>	15	Same	None
<i>Pseudomonas aeruginosa</i>	8	Same	7 (Tetra, AD); 7 (Mino, AD)
<i>Pseudomonas</i> sp.	6	Same	None
<i>Streptococcus faecalis</i>	15	Same	1 (Erythro, AD); 2 (Tetra, AD and MD); 2 (Meth, MD); 7 (Pen, all methods)
<i>Staphylococcus epidermidis</i>	5	AST, TD	None
<i>S. pneumoniae</i>	5	Same	1 (Pen, TD)
<i>S. pyogenes</i> (A)	5	Same	None
<i>S. mitior</i>	5	Same	None
<i>S. agalactae</i> (B)	5	Same	None
<i>Listeria monocytogenes</i>	5	Same	None
<i>Providencia stuartii</i>	5	Same	None
<i>Proteus mirabilis</i>	5	Same	2 (Amp, TD)
<i>Serratia marcescens</i>	5	Same	None
<i>S. liquefaciens</i>	5	Same	None
<i>Enterobacter agglomerans</i>	5	Same	None
<i>E. aerogenes</i>	5	Same	None
<i>Enterobacter hafniae</i>	5	Same	None
<i>Shigella</i> sp.	5	Same	None
<i>Salmonella</i> sp.	5	Same	None
<i>Enterobacter cloacae</i>	5	Same	None

<sup>a</sup> AD, Agar dilution; MD, microdilution; TD, tube dilution; AB, Autobac; AST, Lederle; other abbreviations as in Footnote a, Table 2.

<sup>b</sup> Instances in which AST MIC was >1 d.i. from indicated MIC.

have been interpreted as susceptible to penicillin.

*S. aureus* showed two discrepancies each for ampicillin and penicillin when AST was compared with microdilution and one each for

methicillin and penicillin when compared with agar dilution.

Problems of agreement were observed with the enterococci (*Streptococcus faecalis*). Of the discrepancies noted in Table 3, the most numer-

ous were seen with penicillin when AST was compared with microdilution (seven discrepancies) and agar dilution (six discrepancies). Similarly, seven discrepancies were seen when *P. aeruginosa* was tested with tetracycline and minocycline.

Table 4 presents preliminary data that test the versatility of the AST strip. Fastidious organisms such as *H. influenzae*, pathogenic *Neisseria*, and gram-negative anaerobic rods were tested with the modifications of media and incubation conditions as indicated. In all cases, the AST results were within 1 d.i. of the broth dilution results.

### DISCUSSION

The continuing use of toxic antimicrobial agents, the appearance of bizarre opportunistic protista, and the increasingly sophisticated therapeutic modalities in infectious disease have dictated the need for a rapid, economical, and accurate MIC. The product known as the AST appears to fulfill these specifications. The AST, a multiwelled tray containing diluted antibiotics, is not a new concept in antimicrobial susceptibility testing. Rather, it is a hybridization of the cumbersome broth dilution test and the microdilution MIC test. The uniqueness of the system is that the significant labor-draining steps of an MIC determination have been circumvented; that is, the preparation of an antibiotic solution and its subsequent dilution in tubes, wells, or agar plates. To determine an MIC, the technologist only standardizes the bacterial inoculum in fresh broth and adds it to the multiwelled chamber before incubation.

The joint study to evaluate this device initially focused on the biological parameters associated with the performance of the test. Once defined, these parameters were incorporated as a routine procedure to test the AST with a limited number of microorganisms commonly encountered in the clinical laboratory.

The test conditions, although predictable from previous investigations (3-5, 7, 8), revealed that the AST was susceptible to inoculum effect as well as the vagaries of the growth medium. It was observed that accurate MICs could be read visually as early as 5 to 6 h after inoculation. This may be a function of the ability to pick up the strip, shake it, and observe turbidity through the side of the chambers. Examining the wells directly through the top with transmitted light did not permit visualization of the turbid end points, as is the case with the microdilution MIC procedure after overnight incubation.

The limited clinical trial of the AST suggested that there was satisfactory interpretive agreement between the AST and other accepted susceptibility test methods. (However, when variation occurred, the AST MIC was usually of a higher value, that is, more resistant than the other methods.) When numerous major discrepancies were observed, such as with penicillin and *S. faecalis*, it was assumed that there were errors in the manufacture of the product. In some cases, the pellet of dried antibiotic adhered to the cover, and a portion of it was lost when the cover was removed. A more extensive clinical trial will quantitate the methodological agreement.

The versatility of the AST for fastidious microorganisms was assessed. *H. influenzae* MICs could be read in 18 h if the nutrient medium was supplemented with supplement C (Difco), yeast extract, or 10% Fildes reagent. The MICs generated showed no significant variation when compared with broth dilution techniques.

The pathogenic *Neisseria* species could be tested similarly. The MICs of some fast-growing isolates of *N. meningitidis* could be read in 6 h. *N. gonorrhoeae* required 18 h of incubation for the assessment of results.

Two genera of anaerobic gram-negative rods were tested. Preliminary data on seven strains

TABLE 4. Summary of limited clinical testing of the AST method with fastidious bacteria

Organism	No. of isolates	Method <sup>a</sup>	Environmental/nutritional supplementation	Discrepancies
<i>Haemophilus influenzae</i>	10	BD, AST	Capneic incubation, yeast extract (1%), and hemoglobin (1%) added to M-H broth	None
<i>Neisseria gonorrhoeae</i>	5	BD, AST	Same	None
<i>N. meningitidis</i>	5	BD, AST	Same	None
<i>Fusobacterium sp.</i>	3	BD, AST	Anaerobic incubation (GasPak), thioglycolate broth (hemin-menadione supplement)	None
<i>Bacteroides fragilis</i>	4	BD, AST	Same	None

<sup>a</sup> BD, Broth dilution.

tested against tetracycline, ampicillin, penicillin, and clindamycin indicated that if a thioglycolate broth inoculum was added to the strip and the cover was left loose, then MIC results determined after anaerobic incubation in a GasPak jar consistently agreed with the tube dilution results.

Several features of the AST make it a desirable addition to the clinical microbiology laboratory. They are as follows.

(i) **Rapidity of processing and reading.** An AST MIC can be set up in less than 5 min. A 6-h reading is possible with the fast-growing organisms.

(ii) **Accuracy and reproducibility.** The AST showed excellent interpretative agreement with the commonly used dilution tests. Repetitive MICs on replicate AST strips indicated that the test results were reproducible.

(iii) **Widespread applicability.** The AST test provides the capability for any laboratory, regardless of size or sophistication, to perform MICs. No storage of antibiotic reference powder, no critical weighing of antibiotics, and no serial dilution procedures are necessary.

(iv) **Versatility.** Preliminary experiments show that with minor modifications of nutrient and/or incubation environment, MICs may be performed on fastidious microorganisms such

as the anaerobic bacteria, *Haemophilus*, and *Neisseria*.

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