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Relationship of Early Readings of Minimal Inhibitory Concentrations to the Results of Overnight Tests

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Broth dilution minimal inhibitory concentration (MIC) readings were compared after different incubation periods and with different inoculum concentrations. The purpose was to determine the best conditions for obtaining early results as close as possible to overnight readings. Initially, 76 antibiotic-organism combinations were tested using the International Collaborative Study technique and inoculum and were read after 3, 8, and 18 h of incubation. Approximately 28% of tests showed fourfold or greater increases in MICs after 18 h of incubation compared with the 3-h readings. No overnight MICs were lower than early readings. MICs of single antibiotics against seven organisms were also read with an automatic particle counter to confirm the validity of the visual readings. Experiments were made to determine whether inoculum manipulation could reconcile the differences between 3- and 18-h MIC results. One hundred and eight organism-antibiotic combinations were tested comparing 3-h MIC readings using an inoculum of 10^7 organisms per ml with overnight readings using 10^5 per ml. In 71 cases, readings with both inocula were within the range tested and 57 (86%) were within $\pm 1 \log_2$ of each other and followed an approximately normal distribution. Improved comparability between early read and overnight MICs thus may be achieved by inoculum manipulation, and this may be a suitable approach in the future development of automated procedures.

The majority of fully quantitative antimicrobial susceptibility tests undertaken in clinical and investigative work have involved overnight reading of minimal inhibitory concentrations (MICs) of the antimicrobial agent. This reading interval was recommended for the agar and broth dilution methods proposed as reference procedures by the International Collaborative Study (ICS) group (3).

Recently, there has been increasing interest in the development of rapid, mechanized and automated procedures for susceptibility testing which could provide results on the same day the test is set up (2, 7, 11, 15). To be fully practicable for routine purposes and to have major clinical advantage over present methods, these procedures should permit readings at a maximum of 5 h. Also, the readings should correlate closely with the results derived from traditional overnight tests.

It has long been known that MICs may increase with duration of incubation (4, 6, 8, 10, 14). This is particularly striking with organisms producing extracellular antibiotic inactivating enzymes, such as β-lactamase-producing staphylococci (12), with antibiotics which deteriorate during incubation (14), and when high mutation rates to resistance exist. However, the overall magnitude of differences between early and late MIC readings have not been studied quantitatively or in detail.

The immediate purpose of this study was to compare MIC readings made after different incubation periods with a range of organism-antibiotic combinations, and to determine whether early and overnight results could be brought into conformity by mathematical or technical manipulations. The ultimate purpose was to assist in the selection of conditions for early reading automated systems.

MATERIALS AND METHODS

Media. Test organisms were initially grown in Trypticase soy broth (BBL) enriched with 1% yeast extract (Difco). Mueller-Hinton broth (Difco) and Eugon broth (BBL) were used for broth dilution studies. Eugon broth, containing 0.2% KNO_3 and filtered to free it of any contaminating particles, was used for the experiments involving mechanical bacteria counting.

Antibiotics. Antibiotic diagnostic powders were supplied by Canalco. From these, stock solutions were made at concentrations of 640, 1,280, or 2,560 μg/ml

and kept frozen at −20 C until used. Once thawed they were not refrozen. Stock solutions were diluted to working concentrations with Mueller-Hinton or Eugon broth. The antibiotics studied were: ampicillin (A), cephalothin (C), chloramphenicol (CH), kanamycin (K), methicillin (M), polymyxin B (P), and tetracycline (T).

Organisms. The studies were made with 21 strains, which are listed below along with the antibiotics they were resistant to, as determined by the results of an 18-h MIC (3) or disk diffusion technique (5). Only staphylococci were tested with methicillin. (i) *Staphylococcus aureus* was resistant to A and P; (ii) *S. aureus* to A, CH, M, P, and T; (iii) *S. aureus*, fully susceptible; (iv) *Enterococcus* to K, P, T; (v) *Staphylococcus epidermidis* to K; (vi) *Escherichia coli*, fully susceptible; (vii) *E. coli* to A, T; (viii) *Klebsiella* to A, K, T; (ix) *Pseudomonas aeruginosa* to A, C, CH, K, T; (x) *P. aeruginosa* to A, C, CH, K, T; (xi) *Proteus inconstans* to A, C, CH, K, P, T; (xii) *Acinetobacter* to A, C, CH, P; (xiii) *S. aureus* to P; (xiv) *S. aureus* to A, M, P, T; (xv) *Enterococcus* to CH, T; (xvi) *S. epidermidis* to A, K, P, T; (xvii) *E. coli* to T; (xviii) *Klebsiella* to A, K, T; (xix) *P. aeruginosa* to A, C, CH, T; (xx) *P. inconstans* to C, CH, P, T; and (xxi) *Acinetobacter* to A, C. An additional three recent isolates of *P. aeruginosa* and four of *E. coli* were added for studies on the effects of inoculum variation.

Susceptibility test procedures. The initial series of tests involved the first 12 strains and compared the results of MIC readings with a single inoculum after different periods of incubation. They were performed according to the ICS protocol for broth dilution tests (3). The inoculum was derived from a logarithmically growing culture adjusted to the density of the barium sulfate standard used for the Food and Drug Administration diffusion test (5). This suspension was then diluted 10^{-2} in broth. The final bacterial concentration in the test was approximately 5×10^5 to 5×10^6 per ml. Three controls were included: a growth control without antibiotic; an inoculated tube, immediately refrigerated as a control on the turbidity contributed by the original inoculum; and an uninoculated broth tube, incubated with each test series. Tests were read at 3, 8, and 18 h by comparing the turbidity in the tubes with that of the inoculated, refrigerated control using indirect lighting against a dark background. Two observers read each test and the MIC was interpreted as the lowest concentration of antibiotic which gave no turbidity visible to the naked eye, other than any contributed by the initial inoculum. If there was a discrepancy between the results recorded by two readers in the early reading, the end point that was closer to the overnight result was taken as the true value. Tests of seven organisms with single antibiotics were also read with an automatic particle counter (Technicon Auto-counter equipment) to check the validity of the visual readings. Growth was considered to have occurred if there was a doubling of the original inoculum at the time of reading. At each time interval, visual MICs were read, and 1 ml from the test series was added to sampling cups containing 0.25 ml of 10% formalin solution to prevent further growth before automatic reading.

Tests comparing different inocula were made in Eugon broth. The inoculum was first equated in turbidity to the barium sulfate standard used for the Food and Drug Administration diffusion test procedure (5) and was then diluted 10^{-1} and 10^{-3} in Eugon broth to yield inocula of approximately 10^7 and 10^5 per ml. Otherwise, the ICS protocol was followed. MIC readings with the lighter inoculum were made at 18 h, and with the heavier inoculum at 3 and 18 h. All organisms were tested against selected antimicrobials to yield 108 organism-antibiotic combinations. End points were determined by two or occasionally three readers. Any disagreements were reviewed and, if necessary, resolved by a third reader. In several instances, the results were checked with the mechanical particle counter.

RESULTS

A total of 76 antibiotic-organism combinations were first tested with the inoculum recommended by the ICS, and the results were read visually at intervals as described above. In 10 instances, the organisms were highly resistant and end points were above the range of dilutions tested at all reading times; in six instances, all readings were below the range tested. These 16 results were excluded. A comparison of 3-h and overnight readings of the remaining 60 tests are shown in Table 1 and expressed as the ratio of the overnight to the 3-h MIC. In 39 cases (line 1), end points at both time intervals were within the range of antibiotic concentrations tested. In 10 cases (line 2), the 3-h MIC was within range but the overnight MIC was above the highest concentration used. In 11 cases (line 3), the 3-h MIC was below the range tested but the overnight MIC was within the range. Most MIC results were higher after overnight incubation and in some cases the changes were large. No decreases in MIC were encountered after over-

TABLE 1. *Comparison of overnight to 3-h readings with ICS broth dilution method*

Determinants	Ratio of 18-h to 3-h readings							
	1	2	4	8	16	32	64	128
3- and 18-h MICs within range tested	10	18	7	1	2	1	0	0
18-h MIC[a] above range tested	–[b]	6	2	1	1	0	0	0
3-h MIC[a] below range tested	–[c]	0	3	2	1	1	1	3

[a] Ratios are thus equal to or greater than indicated figures.

[b] In 10 cases, both MICs were above the range tested and results were excluded.

[c] In 6 cases, both MICs were below the range tested and results were excluded.

night incubation. Approximately 28% of tests in which both readings were within range showed fourfold or greater increases in MIC after 18 h of incubation.

When tests read after 8 h of incubation were compared with overnight results, there were similar but less marked discrepancies. Among 51 tests in which at least one of the readings was within range, identical MICs were obtained in 16, twofold higher MICs were observed with the overnight reading in 20, fourfold in 11, and between eight- and 128-fold in the remainder.

Eleven antibiotic-organism combinations which gave discrepancies of two- to 128-fold between the 3-h and overnight readings with Mueller-Hinton medium were also tested with Eugon broth. Nine yielded discrepancies of similar magnitude to those with Mueller-Hinton broth. The same series of tests also compared 3-h readings in static and shaken cultures in Eugon broth. Shaking did not eliminate the discrepancies.

Visual end points at 3 h were inevitably more subjective than overnight readings because of the light growth density. To determine whether the findings given in Table 1 were seriously influenced by this problem, seven combinations which showed differences between early and late MICs were also read with a mechanical particle counter. In each case, the mechanical counter yielded an MIC reading equal to or within a twofold dilution of the 3-h visual reading (Table 2).

The organisms and antibiotics involved in discrepancies of fourfold or more between overnight and 3-h readings are shown in Table 3. All the antibiotics tested, and all except one of the organisms, were involved in at least one such discrepancy, and no single factor could be applied to correct for results with particular antibiotics.

The possibility of equating 3-h readings more closely to the results of overnight tests by manipulating the inoculum size was investigated by making parallel tests using inocula of approximately 10^5 and 10^7 per ml. One hundred and eight organism-antibiotic combinations were tested in this way, including the full range of tests with organisms 13–21. Eighty-six combinations had a least one result within the range of antibiotic concentrations tested. The relationship of the 3-h readings with the inoculum of 10^7 per ml to those of overnight readings at 10^5 per ml with these strains is shown in Table 4. There were 71 pairs of readings within the range tested (line 1). These showed an approximately normal distribution with a slight shift towards higher MIC results with the 3-h reading

TABLE 2. *Comparison of 3-h MICs determined by particle counting and by visual reading*

Organism	Antibiotic	3-h MIC (μg/ml)	
		Mechanical	Visual
Staphylococcus aureus (i)	Ampicillin	8	8
S. aureus (ii)	Ampicillin	0.25	0.12
S. aureus (iii)	Ampicillin	≤0.06	0.12
Pseudomonas aeruginosa (x)	Kanamycin	128	128
Klebsiella (viii)	Chloramphenicol	4	2
Escherichia coli (vii)	Chloramphenicol	4	2
E. coli (vi)	Chloramphenicol	4	2

TABLE 3. *Antibiotic-organism combinations showing discrepancies of fourfold or more between 3-h and overnight MICs with ICS broth dilution method*

Organism	Antibiotic	Discrepancies[a] (-Fold)
Staphylococcus aureus (i)	T	4
	C	8
	K	8
	A	16
S. aureus (ii)	K	4
	A	16
S. aureus (iii)	CH	4
	T	4
Klebsiella (viii)	CH	4
	P	4
Proteus inconstans (xi)	CH	4
	T	4
Escherichia coli (vi)	K	4
	P	8
Pseudomonas aeruginosa (ix)	P	4
	T	4
	CH	8
P. aeruginosa (x)	K	4
	T	32
Staphylococcus epidermidis (v)	P	16
	CH	32
	A	64
	K	128
	M	128
Enterococcus (iv)	T	16
Acinetobacter (xii)	P	128

[a] Comparison of overnight to 3-h readings.

TABLE 4. *Comparison of overnight MIC readings with inocula of 10^5 per ml to 3-h readings with inocula of 10^7 per ml*

Determinants	Ratio of 18-h to 3-h readings							
	0.03	0.125	0.25	0.5	1	2	4	8
3- and 18-h MICs within range tested		2	5	20	32	9	2	1
One of the two MICs above the range tested[a]	1	1	2	6	—[b]		1	
One of the two MICs below the range tested[a]				4				

[a] Ratios calculated by considering MIC readings of, e.g., >128 as 256, and <0.25 as 0.125.

[b] In 22 cases, both MICs were above the range tested and were excluded.

of tests inoculated with 10^7 per ml. The mode was at the point of equivalence between tests with the two inocula. Approximately 86% of results were within ± 1 $\log_2$ dilution of each other.

DISCUSSION

The data from these experiments confirm that the MIC of many organism-antibiotic combinations increases with the duration of incubation and that 3-h readings may be as much as 100-fold lower than results of overnight tests. This phenomenon has posed difficulties in developing early reading automated susceptibility testing procedures intended to provide results with high levels of comparability to traditional overnight tests. The approach adopted to resolve this difficulty in two automated systems (7, 11) has been to reduce the concentration of antibiotic in the test system to achieve maximum comparability; however, our findings suggest that this is unlikely to be fully successful even though it may bring reproducibility of categorizations of susceptibilities to acceptable levels with most organism-antibiotic combinations.

The development of early read automated tests would be rationalized and facilitated if comparability to traditional methods could be improved by other manipulations, so we tested the effect of increasing the inoculum for the early readings by 10^2. The results reported are encouraging and we believe the approach merits extended investigation with automated equipment and a wider range of inocula. A major benefit of this approach would be that the selection of concentrations of antibiotics for early read tests could be directly derived from quantitative data and concepts which have been developed cumulatively over the years.

Some of the reasons for discrepancies between early and late readings have already been considered in the introduction to this paper. The generally higher MICs at 3 h with increased inocula may similarly involve several factors. Nonspecific absorption of the antibiotic by the organisms (1, 4, 6, 9) may lower the effective concentration of antibiotic to subinhibitory levels. Resistance due to antibiotic-inactivating enzymes may be expressed earlier with a heavier inoculum, both because of increased concentration of enzyme in the inoculum and because of greater production during incubation (1, 13). If the inoculum contains mixtures of resistant and susceptible cells for mutational or any other reasons, the resistant population will be more rapidly expressed with a heavier inoculum.

These factors make it improbable that complete correspondence of results between early read tests with a heavy inoculum and overnight tests will be achieved. Nevertheless, our data suggest that the approach adopted may yield the closest correlation.

Finally, it should be stressed that overnight incubation for MIC determinations is an artificial situation itself and probably at variance with what happens in vivo. For example, several antibiotics deteriorate during 18 h of incubation, yet they may be given to the patient every 4 to 6 h. Similarly, intact host defenses interact with the action of chemotherapeutics on accessible organisms in vivo and cannot be mimicked in vitro. Nevertheless, until more information is available, the overnight MIC performed under standardized conditions must remain the yardstick against which other dilution procedures, including early reading automated test results, are judged.

LITERATURE CITED

1. Cooper, K. E. 1963. The theory of antibiotic inhibition zones, p. 1-86. *In* F. Kavanagh (ed.), Analytical microbiology. Academic Press Inc., New York.
2. Deblanc, H. J., Jr., P. Charache, and H. N. Wagner, Jr. 1972. Automatic radiometric measurements of antibiotic effect on bacterial growth. Antimicrob. Agents Chemother. **2**:360-366.
3. Ericsson, H. M., and J. C. Sherris. 1971. Antibiotic sensitivity testing. Report of an international collaborative study. Acta Pathol. Microbiol. Scand. Sect. B **217**(Suppl.):1-90.
4. Erlanson, P. 1951. Determination of the sensitivity in vitro of bacteria to chemotherapeutic agents. Acta Pathol. Microbiol. Scand. **85**(Suppl.):1-165.
5. Federal Register. 1972. Rules and regulations. Antibiotic susceptibility discs. Fed. Reg. **37**:20525-20529.
6. Guthrie, F. 1958. A study of methods for determining sensitivity of bacteria to antibiotics and the factors influencing differences in results. Am. J. Med. Tech-

nol. 24:109-119.
7. Isenberg, H. P., A. Reichier, and D. Wiseman. 1971. Prototype of a fully automated device for the determination of bacterial antibiotic susceptibility in the clinical laboratory. Appl. Microbiol. 22:980-986.
8. Jackson, G. G., and M. Finland. 1951. Comparison of methods for determining sensitivity of bacteria to antibiotics in vitro. Arch. Int. Med. 88:446-460.
9. Lindberg, D. A. B., and G. T. Stewart. 1966. Exercises in the logistics of chemotherapy, p. 168-176. Antimicrob. Agents Chemother. 1965.
10. Sherris, J. C., A. L. Rashad, and G. A. Lighthart. 1967. Laboratory determination of antibiotic susceptibility to ampicillin and cephalothin. Ann. N.Y. Acad. Sci. 145:248-267.
11. Thornsberry, C., T. L. Gavan, J. C. Sherris, A. Balows, J. M. Matsen, L. D. Sabath, F. D. Schoenknecht, L. D. Thrupp, and J. A. Washington II. 1975. Laboratory evaluation of a rapid, automated susceptibility testing system: report of a collaborative study. Antimicrob. Agents Chemother. 7:466-480.
12. Sutherland, R., and G. N. Rolinson. 1964. Characteristics of methicillin-resistant staphylococci. J. Bacteriol. 87:887-899.
13. Waisbren, B. A., C. Carr, and J. Dunnette. 1951. The tube dilution method of determining bacterial sensitivity to antibiotics. Am. J. Clin. Pathol. 21:884-891.
14. Wick, W. E. 1964. Influence of antibiotic stability on the results of in vitro testing procedures. J. Bacteriol. 87:1162-1170.
15. Wyatt, P. J. 1969. Identification of bacteria by differential light scattering. Nature (London) 221:1257-1258.