ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1977, p. 1021-1026

Vol. 11, No. 6
Printed in U.S.A.

# Manner and Meaning of Susceptibility Testing of Ampicillin-Resistant *Haemophilus influenzae*

BARRY M. GRAY, CAROL A. HUBBELL, AND HUGH C. DILLON, JR.*

*Department of Pediatrics, University of Alabama in Birmingham, School of Medicine, Birmingham, Alabama 35294*

Received for publication 31 January 1977

We examined ampicillin-resistant strains of *Haemophilus influenzae* to compare the percentage of resistant organisms in each strain with the susceptibility to ampicillin by an agar dilution method. Using an inoculum of $10^4$ colony-forming units, the minimal inhibitory concentration (MIC) increased with the percentage of resistant organisms in the strain. Laboratory-manipulated strains composed of different proportions of a susceptible and a resistant strain behaved similarly. The survival of isolated colony-forming units (colony MIC) was then determined by spreading inocula over the surface of a set of MIC plates, resulting in separation of individual colonies. This modification of the susceptibility test to the colony level gave end points that were clear and reproducible and that did not vary with changes in incubation time or temperature. True differences in susceptibility among strains were demonstrated by this method, whereas results of the conventional MIC test may reflect only the number of resistant organisms present in the inoculum.

Since the emergence of ampicillin-resistant strains of *Haemophilus influenzae*, reliable susceptibility testing of this organism has become a matter of grave importance. Reports concerning susceptibility patterns, as well as case reports, often describe ampicillin-resistant *H. influenzae* in terms of a minimal inhibitory concentration (MIC) as though this were a standard and meaningful laboratory measure. In fact, the fastidious nature of *H. influenzae* necessitates the modification of standard methods merely to obtain growth adequate for testing purposes. Variables such as inoculum size, media, and incubation conditions may all influence results. Among ampicillin-resistant strains, the important factor appears to be the size of inoculum used, regardless of whether agar or broth dilution techniques are used (3, 12). Inoculum size refers to the total number of colony-forming units (CFU) placed into a broth or spotted onto an agar plate. A larger inoculum yields a higher MIC. Cultures of ampicillin-resistant *H. influenzae*, however, are not homogeneous. Resistance is conferred by a plasmid coding for a β-lactamase (10). A resistant strain may contain individuals that do not bear the plasmid and are consequently susceptible to ampicillin at the usual concentrations. Spontaneous loss of the plasmid may occur frequently under laboratory conditions (1). We have also observed this phenomenon and found it to affect the MIC result.

We hypothesized that, inoculum size and other conditions being equal, the MIC of a strain depends on the proportion of β-lactamase-producing individuals in that strain at the time of testing. Our experiments show this to be the case; furthermore, true differences among strains were demonstrated by a method that tests the susceptibility of individual CFU.

## MATERIALS AND METHODS

**Organisms.** We have recently examined 271 strains of *H. influenzae* by agar dilution and disk diffusion tests for susceptibility to ampicillin and two cephalosporins (B.M. Gray, C.A. Hubbell, and H.C. Dillon, Jr., manuscript in preparation). All were clinical isolates recovered from patients at The Children's Hospital, Birmingham, Ala., in 1971, 1975, and 1976. Twenty-six strains were ampicillin resistant, of which 14 were selected for further study. Seven were type b (strains 3, 4, 6, 7, 8, 9, and 13); the remainder were nontypable. Strains were preserved in skim milk at −60°C.

**Agar dilution techniques.** (i) Strains were recovered from storage on Levinthal agar made with washed human erythrocytes (6, 13). Five to six colonies were incubated until growth appeared (3 to 6 h) in brain heart infusion broth (Difco) supplemented with 8 μg of hemin (type 1, bovine; Sigma Chemical Co.) per ml and 5 μg of nicotinamide adenine dinucleotide (β-NAD, grade III; Sigma Chemical Co.) per ml. Samples (0.1 and 0.2 ml) of this culture were inoculated into prewarmed broths and grown for 2 to 3 h to ensure log-phase growth. Growth was monitored by colony count and optical density; broth

cultures of optical density 0.07 at 590 nm yield $10^7$ to $10^8$ CFU/ml.

(ii) MIC plates were prepared according to the ICS recommendations (4), using ampicillin (anhydrous standard; Wyeth Laboratories) in Levinthal agar poured in 150-mm petri dishes. Plates were used within 24 h.

(iii) The conventional agar dilution method was done using a replicator like that of Steers et al. (11); the bacterial concentration was adjusted such that each pin delivered $10^4$ CFU to 5-mm spots on the MIC plates. Plates were incubated in candle jars at 37°C for 18 h. The MIC was read as the lowest dilution of antibiotic to completely inhibit growth.

(iv) The MIC of CFU, hereafter referred to as the "colony MIC," differs from the conventional MIC in that rather than inoculating multiple strains as discrete spots of $10^4$ CFU on the plates, the inoculum of each strain tested is spread over the entire surface of a set of MIC plates, resulting in separation of individual colonies. An inoculum of $10^3$ CFU in 0.1 ml, spread with a bent glass rod and turntable, proved suitable. Duplicate sets of MIC plates were incubated in candle jars, one set at 37°C and the other at 35°C. The colony MIC was read as the lowest dilution of ampicillin to inhibit growth (see also Fig. 6). One to 10 colonies (less than 1% of the inoculum) on the last plate exhibiting growth were disregarded. Plates with few or no colonies were reincubated and examined at 24 and 36 h.

**Determination of percentage of resistant organisms.** Ten clinical isolates were grown in broth and diluted to $10^4$ CFU/ml in 10% brain heart infusion broth-physiological saline. Samples of 0.1 ml were spread, as above, on Levinthal medium (to grow the total population) and on Levinthal medium containing 2 μg of ampicillin per ml (to select the resistant organisms). The percentage of resistant organisms was calculated from colony counts of the two plates (in duplicate) (Fig. 1). The conventional MIC was determined from the same initial broth cultures.

## RESULTS

In the course of testing nearly 300 *H. influenzae* strains for ampicillin susceptibility, we observed several phenomena prompting further investigation. The effect of β-lactamase production by a resistant strain on reducing local ampicillin concentrations, thereby allowing neighboring susceptible colonies to grow (Fig. 2), was one such observation. We also noted that the disk diffusion test, which correlated with resistance by MIC in all cases, exhibited a discontinuous or "double" inhibition zone with many resistant strains (Fig. 3). These observations illustrate two important features of ampicillin-resistant *H. influenzae* strains: resistant strains are composed of both susceptible and resistant individuals; and the presence of resistant organisms permits the growth of susceptible organisms in their immediate vicinity. By spotting a large number of organisms as a unit, the conventional MIC is subject to these influences, collectively called the "inoculum effect."

The proportion of resistant organisms in 10 wild strains ranged from 7 to 100% and the MIC increased correspondingly (Fig. 4). Most strains had 70% or more resistant organisms and gave MICs of 64 μg/ml or greater. The same relationship was reproduced artificially with the nine laboratory-manipulated strains, whose MIC also increased with the percentage of resistant organisms (Fig. 5). Since these nine strains

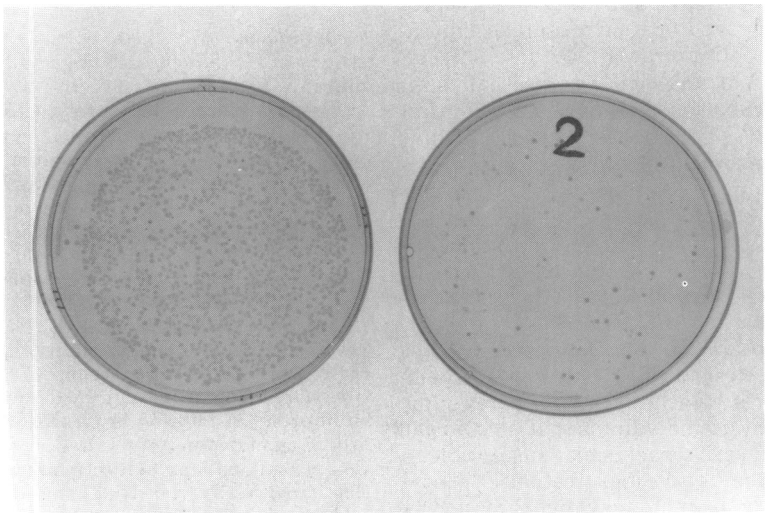



FIG. 1. *Percentage of resistant organisms is calculated from the colony counts of inocula spread on plain agar (left, 1,332 colonies) and on agar containing 2 μg of ampicillin per ml (right, 92 colonies)—for this strain, 7%.*

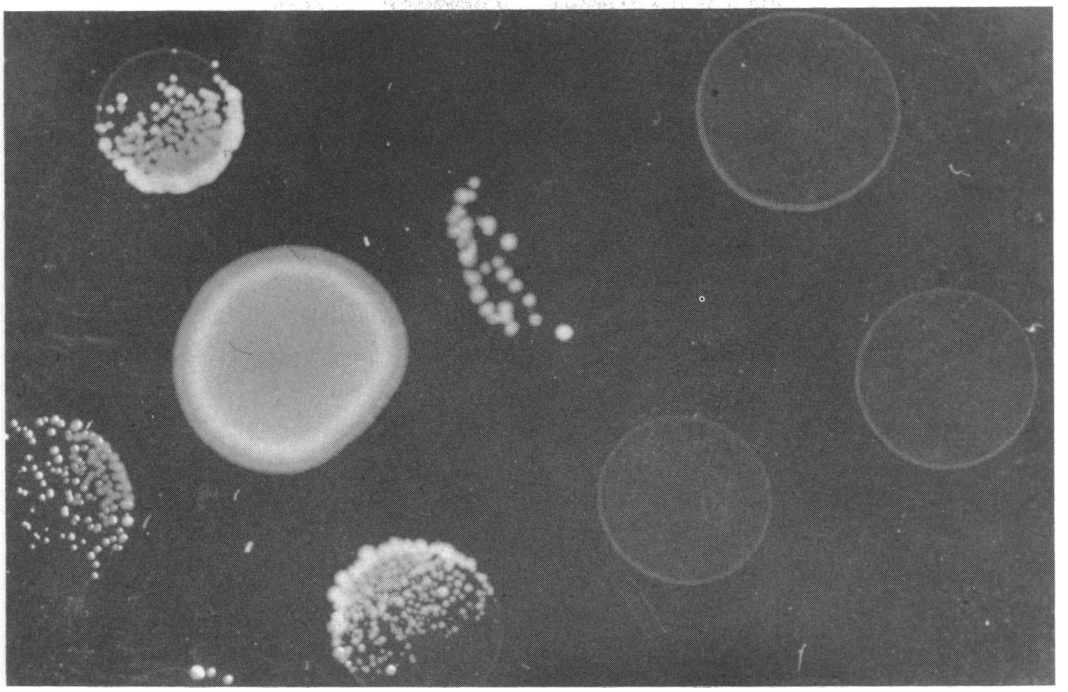


FIG. 2. *Inoculation spots on this 8-μg/ml MIC plate show no growth of susceptible strains to the far right; susceptible colonies adjacent to the resistant strain are capable of growth.*

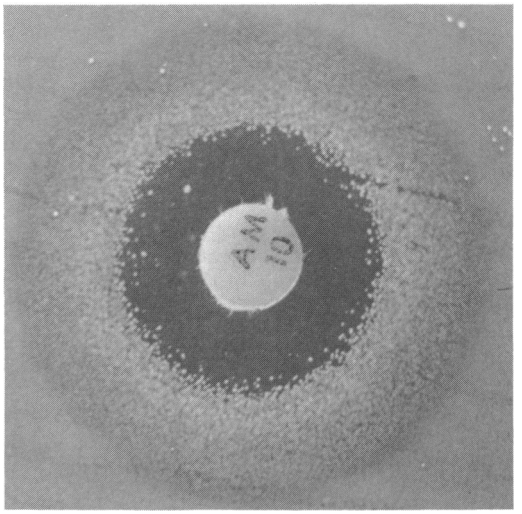



FIG. 3. *Inner ring shows growth of only a few resistant colonies; outer ring approximates the usual susceptibility zone, outside of which all organisms are capable of growth; between these two rings resistant organisms predominate. This effect may not be apparent on opaque media.*

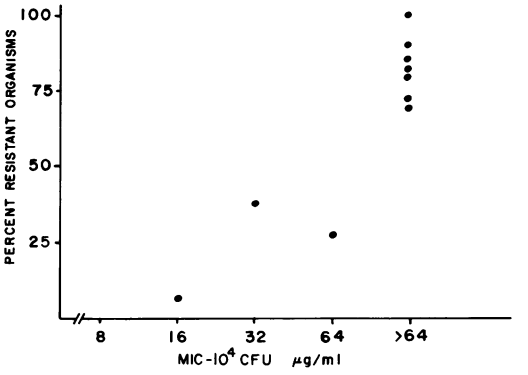



FIG. 4. *Percentage of resistant organisms versus MIC/10⁴ CFU for 10 wild H. influenzae strains.*

were derived from the same parent populations, the effect of inherent strain differences was eliminated in this experiment.

Table 1 summarizes results of conventional MIC and colony MIC determinations and percentage of resistant organisms. Colony MIC values for 12 clinical isolates ranged from 4 to 32 μg/ml. Typical colony MICs are pictured for two strains in Fig. 6. The inhibition of growth on the 4-μg/ml plate clearly marks the end point. Corresponding colony counts displayed graphically showed some decrease at 2 μg/ml. Other strains showed a similar pattern; some had no diminution of growth at the dilution before the end point. End points were all plainly discernible, usually with complete ab-

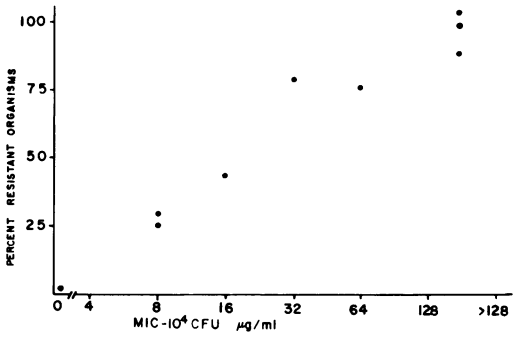



FIG. 5. *Percentage of resistant organisms versus MIC/10⁴ CFU for nine laboratory-manipulated strains.*

sence of growth at the colony MIC; surviving colonies at the end point never exceeded 1% of the inoculum. Reincubation for an additional 6 to 18 h neither increased the number of survivors nor revealed survivors at higher antibiotic dilutions. Colony counts were similar and end points were identical for plates incubated at 35 and 37°C. End points were not altered by preparing inocula from colonies grown on ampicillin-containing agar, and strains retested on different occasions yielded identical results.

## DISCUSSION

Ampicillin-resistant *H. influenzae* are now widely distributed. Chloramphenicol is thus recommended in addition to ampicillin for serious *H. influenzae* infections pending susceptibility tests (2). Clinical laboratories usually perform disk susceptibility testing, which, if properly done, is reliable and provides sufficient information for making clinical therapeutic decisions. Chloramphenicol is discontinued only when *H. influenzae* is unequivocally susceptible to ampicillin. MIC determinations as usually performed serve principally to confirm disk susceptibility results and to ensure quality control in the laboratory. The magnitude of the MIC value may characterize the strain in regard to the size of its resistant population, but does not add clinically useful information or guide decisions on antibiotic dosage. Resistance in any degree demands another antibiotic, such as chloramphenicol, not more ampicillin.

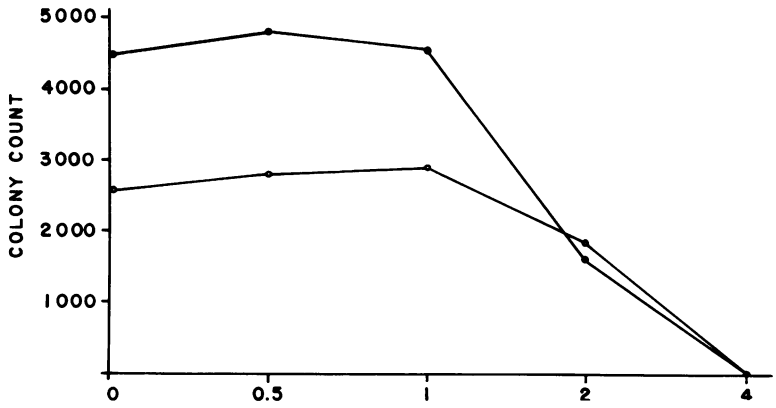
Adequate disk and MIC testing of *H. influenzae* requires modification of standard methods (4). Because of excellent growth, we have found Levinthal agar most suitable. Other media have been used (3, 7, 9, 12), including supplemented Mueller-Hinton agar, which we found unsatisfactory because of lot-to-lot variability and generally poorer growth. Factors such as incubation time (12), atmosphere (4, 8, 12), and temperature (4) also contribute to the result; in general, the MIC is increased by improved growth conditions.

The colony MIC measures the susceptibility of single CFU, thus eliminating the effect of inoculum size and minimizing the influence of environmental factors. This technique is not intended to replace the disk or MIC methods. Rather, it serves to characterize a strain in terms of the susceptibility of its individual colonies. Reasons for differences in colony MIC may be speculated upon. As suggested in the work of Medeiros and O'Brien (10), *H. influenzae* has, to a lesser extent than *Escherichia coli*, a barrier that impedes entry of penicillin into the cell; ampicillin permeability may vary among strains. Another possibility is that strains acquire different β-lactamases or elaborate them in varying quantities.

The colony MIC provides a starting point for examining susceptibility differences at the microbial level, but it is not directly applicable to the situation in vivo, where the inoculum size and constitution of the strain vary considerably. The cerebrospinal fluid may contain $10^5$ to $10^8$ organisms per ml in *H. influenzae* meningitis (5). If a small percentage were resistant, ampicillin might kill some isolated resistant organisms along with the susceptible majority, leaving the remainder to the patient's natural defenses. This may explain anecdotal reports of cures with ampicillin in the face of "resistant" strains. On the other hand, when susceptible organisms are in the minority, they may be protected by their β-lactamase-producing neighbors. In either case, the essential infor-

TABLE 1. *Susceptibility of 14 ampicillin-resistant H. influenzae strains*

| Source | MIC/ CFU | MIC/ $10^4$ CFU | Resistant organisms (%) |
|---|---|---|---|
| Middle ear aspirate | 32 | >64 | 84 |
| Lung aspirate | 32 | >64 | |
| Knee aspirate | 16 | 16 | 7 |
| Cerebrospinal fluid | 8 | >64 | 72 |
| Sputum | 8 | >64 | |
| Throat | 8 | >64 | 100 |
| Lung aspirate | 4 | >64 | 69 |
| Cerebrospinal fluid | 4 | 32 | 38 |
| Blood | 4 | >64 | 80 |
| Tracheal aspirate | 4 | 64 | 26 |
| Sputum | 4 | >64 | |
| Middle ear aspirate | 4 | | |
| Blood | | >64 | 81 |
| Middle ear aspirate | | >64 | 87 |



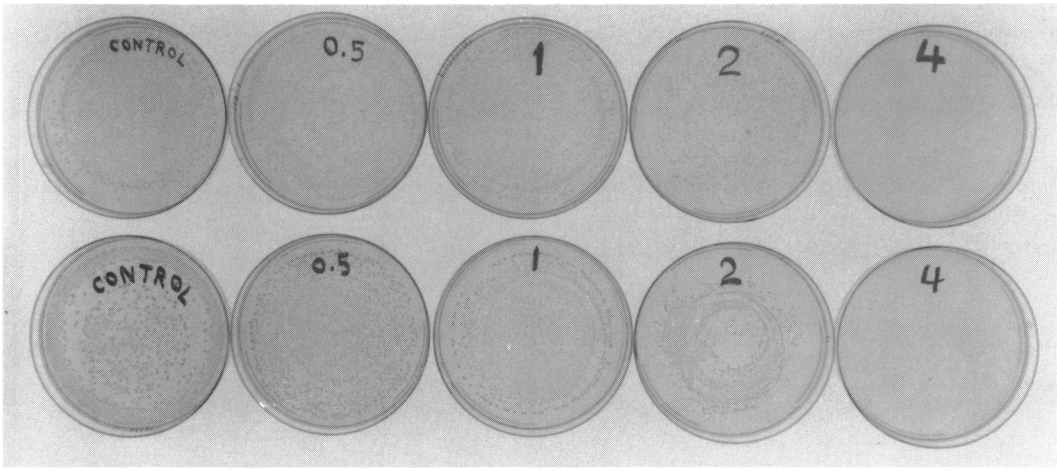




FIG. 6. *MIC of single CFU is determined by spreading inocula of $10^3$ CFU over agar containing serial dilutions of ampicillin. Two strains are pictured with a graph of their respective colony counts (●, upper set; ○, lower set). The MIC is thus 4 μg/ml.*

mation is the presence of resistant organisms, not their proportion or their individual susceptibility, since antibiotic therapy is directed against the entire population. The interaction of resistant and susceptible organisms may alter both laboratory results, as shown by our data, and therapeutic outcome, as evidenced by clinical experience.

## ACKNOWLEDGMENTS

We are grateful to Ann Kimsey and Marcia McCord for preparation of the manuscript, and to Richard Colan and Richard Gunthorpe for the photography.

B.M.G. was supported by Public Health Service postdoctoral fellowship PO 1 HD 00413 from the National Institute of Child Health and Human Development.

## LITERATURE CITED

1. **Caltin, B. W.** 1975. Iodometric detection of *Haemophilus influenzae* beta-lactamase: rapid presumptive test for ampicillin resistance. Antimicrob. Agents Chemother. 7:265-270.
2. **Committee on Infectious Diseases.** 1976. Current status of ampicillin-resistant *Hemophilus influenzae* type b. Pediatrics 57:417.
3. **Emerson, B. B., A. L. Smith, A. L. Harding, and D. H. Smith.** 1975. Hemophilus influenzae type B susceptibility to 17 antibiotics. J. Pediatr. 86:617-620.
4. **Ericson, H. M., and J. C. Sherris.** 1971. Report of the international collaborative study. Acta Pathol. Microbiol. Sect. B, Suppl. 219.
5. **Feldman, William E.** 1976. Concentrations of bacteria in cerebrospinal fluid of patients with bacterial meningitis. J. Pediatr. 88:549-552.
6. **Holt, L. B.** 1961. The growth-factor requirements of *Haemophilus influenzae*. J. Gen. Microbiol. 27:317-322.
7. **Jorgensen, J. H., and P. M. Jones.** 1975. Simplified medium for ampicillin susceptibility testing of *Haemophilus influenzae*. Antimicrob. Agents Chemother. 7:186-190.
8. **Kamme, C.** 1969. Susceptibility in vitro of *Haemophilus influenzae* to penicillin G, penicillin V and ampicillin. Incubation of strains from acute otitis media in air and in $CO_2$-atmosphere. Acta Pathol. Microbiol.

Scand. 75:611–621.
9. **Marks, M. I., and G. Weinmaster.** 1975. Influence of media and inocula on the in vitro susceptibility of *Haemophilus influenzae* to co-trimoxazole, ampicillin, penicillin, and chloramphenicol. Antimicrob. Agents Chemother. 8:657–663.
10. **Medeiros, A. A., and T. F. O'Brien.** 1975. Ampicillin-resistant Haemophilus influenzae type b possessing a tem-type β-lactamase but little permeability barrier to ampicillin. Lancet i:716–719.
11. **Steers, E., E. L. Foltz, B. S. Graves, et al.** 1959. An inocula replicating apparatus for routine testing of bacterial susceptibility to antibiotics. Antibiot. Chemother. (Basel) 9:307–311.
12. **Thornsberry, C., and L. A. Kirven.** 1974. Antimicrobial susceptibility of *Haemophilus influenzae*. Antimicrob. Agents Chemother. 6:620–624.
13. **Turk, D. C., and R. L. May.** 1967. *Hemophilus influenzae*: its clinical importance, p. 114. English Universities Press, London.