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Antibiotic Susceptibility of Clinical Isolates of *Listeria monocytogenes*

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A broth microdilution method was used to measure the minimal inhibitory concentrations (MICs) of the antibiotics most often recommended for treatment of listeriosis. The MICs of ampicillin, penicillin, erythromycin, and tetracycline for 175 strains of *Listeria monocytogenes* were below the approximate MIC breakpoint for susceptible strains as recommended by the National Committee on Clinical Laboratory Standards. Inhibition diameters for 125 strains were measured by the standardized disk method (National Committee on Clinical Laboratory Standards) and compared with the appropriate MIC values. By both methods, strains were susceptible to the above four antibiotics, except for three strains, which were intermediate in susceptibility to penicillin by the disk method. Since the minimal bactericidal concentrations for ampicillin and penicillin significantly exceeded the MICs for these antibiotics, 45 strains were evaluated with ampicillin (5 μg/ml) and gentamicin (1 μg/ml) to compare the synergistic bactericidal effect of the two used in combination and singly. An increased kill of 100-fold was observed with the antibiotics combined in 19 strains after 4 to 6 h and in 40 strains after 24 h. A comparison of results with microdilution in Trypticase soy broth and agar dilution in Mueller-Hinton agar revealed that MICs for gentamicin, kanamycin, and streptomycin were strongly influenced by the media used. The MICs were consistently lower in Mueller-Hinton agar.

Numerous reports have dealt with the clinical and laboratory aspects of *Listeria monocytogenes* infections. Although many authors discussed the susceptibility of these organisms to antibiotics or made recommendations regarding therapy, they did not all agree to any specific optimal regimen for treatment. The antibiotics often suggested include tetracycline (3, 4), penicillin (10, 14), ampicillin (9, 12, 17), and erythromycin (10). Some reports detail the possible advantages of using antibiotic combinations that show synergistic effects against strains of *L. monocytogenes* tested in vitro (8, 13, 15).

The purpose of this study was to determine the in vitro activity of various antibiotics against recent clinical isolates of *L. monocytogenes* from the United States and stock strains representing the known serotypes. Standardized disk diffusion and broth microdilution susceptibility methods were compared. The synergistic activity of gentamicin and ampicillin was studied in strains isolated from neonates.

MATERIALS AND METHODS

Bacterial cultures. *L. monocytogenes* cultures were selected from those submitted to the Center for Disease Control for identification or serotyping. Strains received from 1971 to 1976 are listed in Table 1 by geographical origin and serotype. Clinical isolation sources included: spinal fluid, blood, uterus, pleural fluid, trachael aspirate, heart, eye, nose, ear, liver, spleen, skin, stool, and placenta. *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were included in each experiment as reference strains. *Pseudomonas aeruginosa* ATCC 27853 was also used as a reference strain in some studies.

Reference strains (with serotypes) of *L. monocytogenes* studied included those from: Seeliger 7973 (1a), 5348 (2), 5105 (3), 5214 (4a), and 1071 (4b); Iwanow (5); and Donker-Voet 2459 (1a), 1684 (1b), L461 (3b), F4 (4b), 10 (4c), 21 (4d), 93/65 (4f), 1383 (6), and 1627 (7).

Susceptibility test methods. (i) Disk diffusion method. The standardized diffusion method used was the Bauer-Kirby procedure (1) as described by the Food and Drug Administration (7) and expanded in the tentative report of the National Committee for Clinical Laboratory Standards (NCCLS) (16). Strains were grown at 35°C in Trypticase soy broth (TSB) for 4 to 6 h. The turbidity was adjusted spectrophotometrically, and the inoculum was applied with a cotton swab to plates of Mueller-Hinton agar (MHA) with 5% defibrinated sheep blood.

(ii) Broth microdilution method. For the broth microdilution method, cultures were grown for 18 h at 35°C. One drop of 18-h growth was added to 8 ml of TSB and incubated at 35°C for 4 to 6 h. The turbidity

was adjusted spectrophotometrically to a density equal to a McFarland 0.5 standard, and the growth was then diluted 1:100 in TSB. Serial twofold dilutions of antibiotics were made in tubes with TSB diluent. The inoculum containing approximately 10^6 colony-forming units (CFU) per ml was added to wells in microtiter plates with 0.05-ml dropping pipettes. Equal volumes (0.05-ml) of antibiotic dilutions were added to the microtiter plates with dropping pipettes. Plates were covered with Parafilm and incubated for 18 h at 35°C. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic that prevented macroscopically visible growth after 18 h of incubation. To determine minimal bactericidal concentrations (MBCs), a 0.01-ml portion was removed with a calibrated loop from each well with no visible growth and subcultured onto Trypticase soy agar (TSA). MBC was defined as the lowest concentration of antibiotic that prevented absolute growth on subculture after 18 h of incubation.

(iii) Agar dilution method. The agar dilution technique of Washington (23) was used, with the inoculum prepared as described for the broth microdilution method except that the 4- to 6-h growth was adjusted to a McFarland no. 1 standard and then diluted 1:10. The inoculum was placed in seed plates, and 0.001 ml was delivered by each inoculating rod of a Steers replicator device to MHA plates containing 5% defibrinated sheep blood and antibiotic. The MIC was the lowest concentration of antibiotic at which there was no growth, a very barely visible haze, or one or two discrete colonies.

Synergism studies. Cultures grown (4 to 6 h) in TSB at 35°C and then diluted to contain approximately 10^7 CFU/ml were used as inoculum. Gentamicin and ampicillin were diluted in TSB so that final concentrations of 1 μg of gentamicin per ml (concentration < MIC) and 5 μg of ampicillin per ml (concentration > MIC) were used in the test either singly or in combination. One milliliter of inoculum was mixed with an equal volume of each of the two antibiotics and with the combined antibiotic. Tubes were incubated at 35°C. Colony counts were determined on the initial inoculum, and samples were removed at 4 to 6 h and at 24 h. A sample of 0.1 ml from each tube was transferred, undiluted or in 10-fold dilutions, to a petri dish with TSA. The number of CFU per milliliter were counted after an 18-h incubation. Synergism was defined as a 100-fold decrease in CFU when the antibiotic combination was used as compared with ampicillin alone. Under the experimental conditions used, gentamicin was not bactericidal.

Antibiotics. The antibiotics used in the broth dilution procedure were erythromycin (Abbott Laboratories and Eli Lilly & Co.), ampicillin (Wyeth Laboratories and Pfizer Laboratories), tetracyline (Lederle Laboratories and Upjohn Co.), gentamicin (Schering Corp.), cephalothin (Eli Lilly & Co.), chloramphenicol (Parke, Davis & Co.), kanamycin (Bristol Laboratories), and streptomycin (Pfizer Laboratories). Commercial disks used in the diffusion test included ampicillin (10 μg), penicillin (10 U), erythromycin (15 μg), gentamicin (10 μg), streptomycin (10 μg), and tetracycline (30 μg).

RESULTS

Strains described in Table 1 were grouped according to their MICs, determined by microdilution in TSB, and plotted in cumulative fashion as shown in Fig. 1. All strains were within the susceptible category based on NCCLS approximate MIC breakpoints (expressed in micrograms per milliliter) for erythromycin (≤2.0),

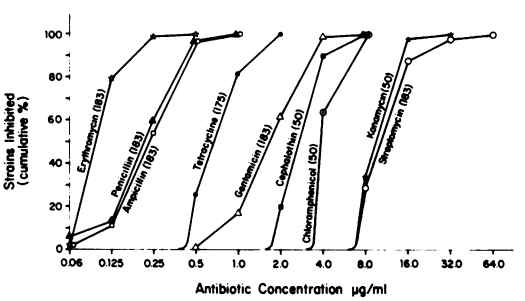

FIG. 1. *Cumulative MIC distribution curves with nine antibiotics. Numbers of strains tested are indicated in parentheses. Results of broth microdilution with TSB.*

TABLE 1. *L. monocytogenes strains by geographical origin and serotype*

Geographical origin	No. of strains by serotype[a]:							
	1a	1b	2	3b	4a	4b	5	7
Area of United States								
New England		4		1		8		
Middle Atlantic	7	5				11		
East North-Central	6	9		2	1	20		
West North-Central	1					4		
South Atlantic	7	14				16		1
East South-Central		3				1		
West South-Central	11	16	1			19		
Mountain	2	2						
Pacific	5	6				5	1	
Foreign	4	1	1			8		1

[a] Selected from those received by the Center for Disease Control from 1971 to 1976.

penicillin (≤1.5), ampicillin (<1.5), tetracycline (≤4.0), chloramphenicol (≤12.5), and cephalothin (≤10.0). Gentamicin MICs were in the sensitive range (≤6.0) for all strains except two, which were intermediate in sensitivity (>6 to <12.5). MICs for two other aminoglycosides, streptomycin and kanamycin, were either intermediate or resistant with all the strains. Stock strains representing all of the *L. monocytogenes* serotypes were also tested with the nine antibiotics, and the MICs were within the same range as those of the clinical isolates.

MBCs for penicillin, ampicillin, streptomycin, and gentamicin were determined with 50 strains. The MBC end points for streptomycin and gentamicin were within a twofold dilution of the MIC. In contrast, the MBCs for penicillin and ampicillin were at least 4 twofold dilutions above the MICs. The effect of the inoculum was evaluated with ampicillin. The number of CFU remaining after 18 h of incubation was influenced by the initial inoculum used (Fig. 2).

Five *L. monocytogenes* strains were diluted to contain 10^7, 10^6, and 10^5 CFU/ml in the initial inoculum. The MICs with ampicillin for all of the inocula were ≤0.5 μg/ml, and the MBCs were all >16 μg/ml. The mean numbers of CFU for the 10^7 inoculum after an 18-h incubation with ampicillin concentrations of 1 to 32 μg/ml were within a 10^6 to 10^7 range. With the 10^6 initial inoculum, the CFU mean for the five strains with these same ampicillin concentrations was in the 10^4 to 10^5 range. With a beginning inoculum of 10^5, the CFU mean was in the 10^2 to 10^3 range.

The number of CFU per milliliter remaining after 18 h of incubation with the NCCLS breakpoint concentrations of ampicillin (1.5 μg/ml) was compared with that remaining after incubation with tetracycline (4 μg/ml) and gentamicin (6 μg/ml) (Table 2). Samples were taken immediately after the antibiotic was mixed with the cells and after 4 and 24 h. The numbers of CFU per milliliter in samples taken immediately after the inoculum was mixed with the three antibiotics were not appreciably different. Samples taken 4 h later showed no substantial decrease in CFU per milliliter with ampicillin or tetracycline, but with gentamicin the CFU per milliliter decreased to 5.7×10^2. At 24 h, the number of CFU per milliliter with tetracycline had decreased only slightly, with ampicillin it had decreased to 3.5×10^4, and with gentamicin it had decreased to <10. Similar results were obtained with five strains tested.

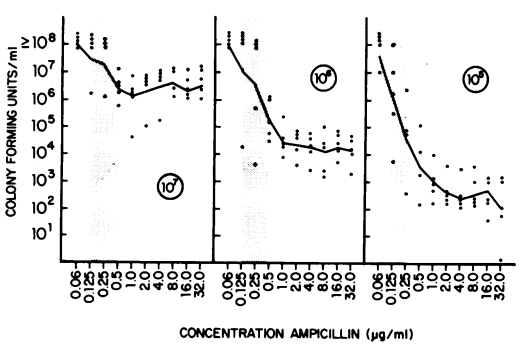

FIG. 2. *Effect of initial inoculum size (10^5, 10^6, or 10^7 CFU) on the viability in various ampicillin concentrations after an 18-h incubation. Shaded areas represent MICs.*

The MICs in microdilution with TSB for 125 strains were compared with inhibition zones by disk diffusion (Fig. 3). When the interpretive standards of the NCCLS were applied, all strains were susceptible by both methods to ampicillin, erythromycin, and tetracycline. All strains were susceptible to penicillin by MIC, although three strains had zone diameters in the intermediate range by the disk procedure. The disk diffusion results with gentamicin indicated susceptibility in all strains tested, although two were considered intermediate by microdilution. Results with the two methods showed least agreement in tests on streptomycin. By the disk diffusion method, all strains were susceptible, except one, which had an intermediate zone; however, all strains were intermediate or resistant by microdilution.

A total of 45 strains isolated from neonates were tested for the synergistic effect of gentamicin and ampicillin. Fifteen strains were tested at three different times, another 15 were tested twice, and the remaining 15 were tested only once. The mean of the different test results was used when strains were tested more than once. Figure 4 is a composite giving the ranges and means of the synergistic results with 45 strains. Nineteen of the strains at 4 to 6 h showed at least a 100-fold decrease in viable cells in the combined antibiotic tube relative to the numbers found with either antibiotic used singly, and 40 of 45 were affected synergistically when sampled at 24 h. All determinations showed increased kill at the times of the early and late readings, except

TABLE 2. *Comparison of killing by ampicillin, gentamicin, and tetracycline with time*

Sample time (h)	CFU/ml on subculture		
	Ampicillin (1.5 μg/ml)	Tetracycline (4 μg/ml)	Gentamicin (6 μg/ml)
Immediate	2.6×10^6	2.2×10^6	1.8×10^6
4	1.3×10^6	1.9×10^6	5.7×10^2
24	3.5×10^4	1.1×10^6	<10

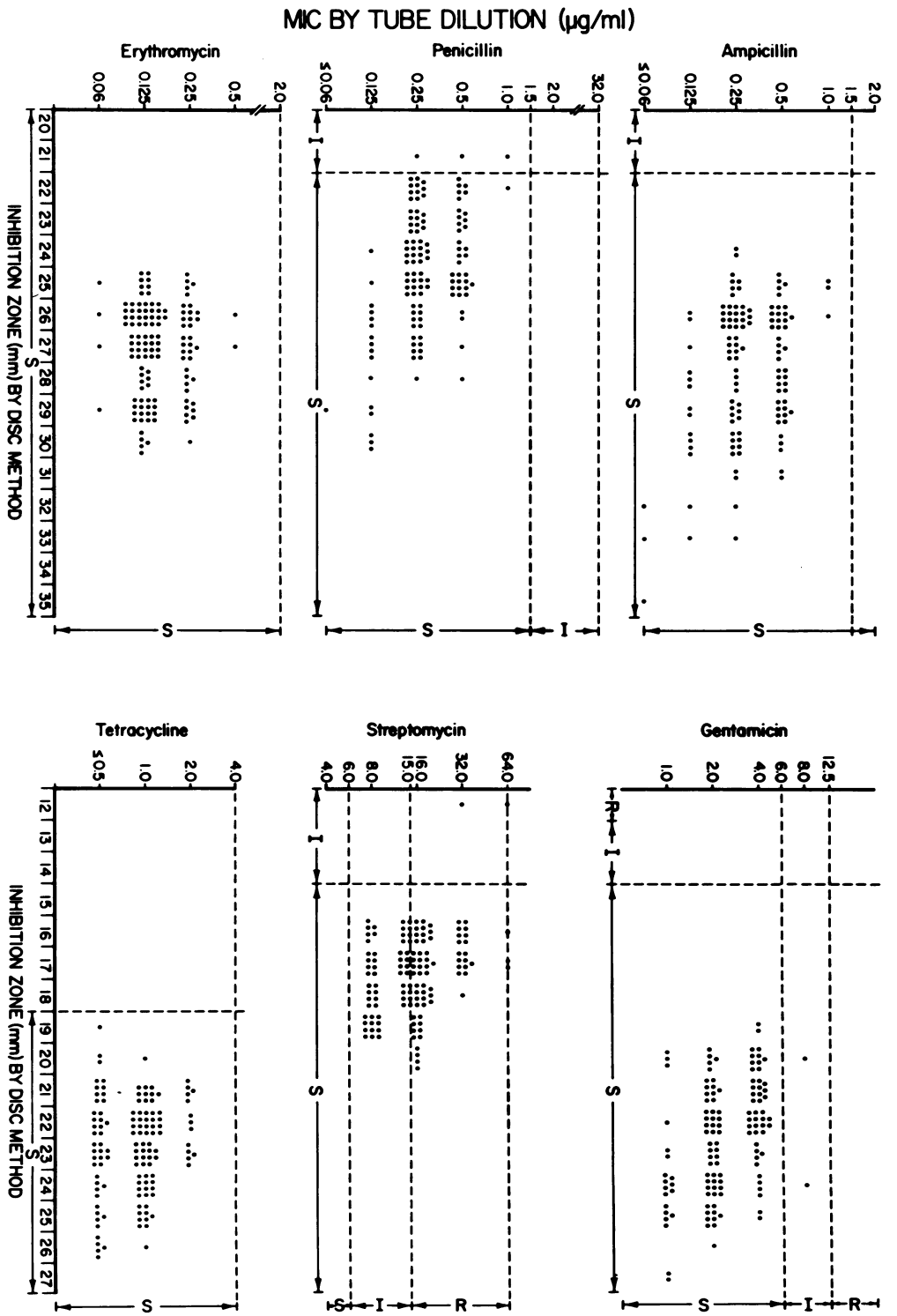

FIG. 3. *Scattergram of MICs and inhibition zone diameters for 125 strains of L. monocytogenes and six antibiotics. The horizontal broken lines represent NCCLS zone diameter interpretive breakpoints, and the vertical broken lines separate the NCCLS approximate MIC breakpoints. S, Susceptible; I, intermediate; R, resistant.*

for one strain that showed a decrease of 0.6 $\log_{10}$ at the early reading in one experiment and one strain that showed a decrease of 0.1 $\log_{10}$ at 24 h.

Table 3 shows a comparison of MICs in MHA and TSB with nine antibiotics. The MIC with a *P. aeruginosa* control strain was 2 μg/ml in TSB and MHA. This strain showed an inhibitory zone diameter of 18 mm with a 10-μg gentamicin disk on MHA. With erythromycin, penicillin, ampicillin, chloramphenicol, and cephalothin, 90% of the end points were within ±1 twofold dilution with the two media. Similar results were obtained for 70% of the strains with tetracycline, whereas end points were consistently ≥2 twofold dilutions lower in MHA than in TSB with the aminoglycosides. These strains were also tested by disk diffusion with the same six antibiotic disks shown in Fig. 3. The inhibition zones were in the same range as those shown for the 125 strains tested previously. Susceptibility interpretations were comparable for all six antibiotics with agar dilution in MHA and with disk diffusion in MHA.

DISCUSSION

Seeliger (20) in 1961 observed that data on in vitro susceptibility of *Listeria* strains varied considerably, but he believed that this variation reflected different test procedures and methods of investigation rather than great differences in susceptibility of the organisms themselves. Using standardized susceptibility tests, we studied a large number of recent clinical isolates and found that they represented a homogeneous and sensitive population in their susceptibility to the antibiotics most often recommended for treatment (ampicillin, penicillin, erythromycin, and tetracycline). Although there have been reports of strains that were "moderately resistant," "relatively resistant," "not fully sensitive," and "resistant" to some of these antibiotics, especially penicillin (2–4, 11, 12, 18, 21), most strains studied have been considered susceptible. The MICs reported here compare well with those of Courtieu et al. (5) in France, who recently tested 136 strains from medical, veterinary, and agricultural sources and reference strains.

In this study a broth dilution rather than an agar dilution method was selected so that both MIC and MBC determinations could be made. Several lots of Mueller-Hinton broth (MHB) were tested, including one containing appropriate concentrations of Ca^{2+} and Mg^{2+} (19); however, growth of *Listeria* strains after 18 h was light, and end points were hard to interpret. Growth in TSB was heavier, and numerous lots of this medium were used during the study period of several years. For disk diffusion, MHA was supplemented with 5% defibrinated blood to enhance growth. MHA and MHB do not appear to be optimal for the growth of *Listeria*.

There have been reported differences in MIC values obtained in MHA, MHB, and TSB for aminoglycosides, tetracyclines, and other antibiotics (6, 19, 23). The differences are attributed to variations in the cation content of the media. Strains of *P. aeruginosa* that can be used to evaluate acceptable performance of Mueller-Hinton media (19) have been described. Because

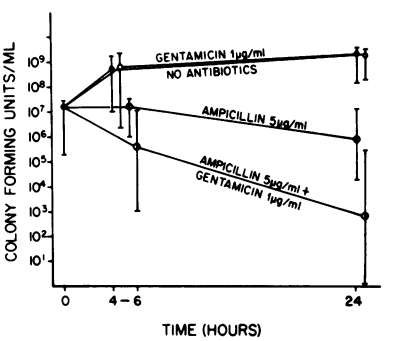

FIG. 4. *Composite of results of 45 strains tested with ampicillin and gentamicin singly and in combination. The range of results is indicated by brackets.*

TABLE 3. *Relationship of MIC in MHA and TSB*

Antibiotic	No. of strains	MIC range (μg/ml) in:		No. of strains at each fold difference in MIC as determined in MHA vs. TSB						
		TSB	MHA	−4	−3	−2	−1	0	+1	+2
Erythromycin	10	0.125–0.25	0.125–0.25	0	0	0	3	7	0	0
Penicillin	10	0.125–0.5	0.025–0.5	0	0	1	3	5	1	0
Ampicillin	21	0.125–1.0	0.25–1.0	0	0	0	11	8	1	1
Tetracycline	10	0.25–1.0	1.0–2.0	0	0	0	0	1	6	3
Gentamicin	21	2.0–4.0	0.125–0.25	13	8	0	0	0	0	0
Kanamycin	21	4.0–16.0	1.0–2.0	12	6	3	0	0	0	0
Streptomycin	21	8.0–16.0	1.0–2.0	1	10	10	0	0	0	0
Chloramphenicol	10	4.0–8.0	8.0–16.0	0	0	0	0	2	7	1
Cephalothin	10	2.0–4.0	2.0–4.0	0	0	0	1	8	1	0

of these reports and the lack of agreement that we found between disk diffusion and microtube dilution results for streptomycin, we evaluated the differences between MICs in TSB and MHA. MHA was pretested for acceptable performance with the ATCC control *P. aeruginosa* strain. The greatest disparity in the end points was with the aminoglycosides. MICs were consistently lower with MHA. Susceptibility interpretation of zones of inhibition with disk diffusion and MICs showed closer agreement for streptomycin when MHA was used in both procedures.

Thornsberry et al. (22) discussed the difficulties of comparing MBCs from different laboratories since MBCs are very method dependent. There have been substantial variations in *Listeria* MBCs reported by workers in the United States. Moellering et al. (15) concluded that penicillin and ampicillin are usually only bacteriostatic against *L. monocytogenes* since the MBCs for these antibiotics were much higher than the MICs, and MBC values were often above levels clinically achievable in blood and almost invariably above those attainable in cerebrospinal fluid. McCracken et al. (13) reported MBCs of ≤ 0.6 μg/ml for 50 strains. These groups used an initial inoculum of approximately 10^5 and defined MBC as complete inhibition of growth on subculture. Gordon et al. (9) used an MBC end point of ≤five colonies on subculture and found MBCs of 6.3 μg/ml for penicillin and ampicillin for three strains and 6.3 and 1.6 μg/ml, respectively, for one strain. They, therefore, postulated that penicillin and ampicillin were bactericidal for *L. monocytogenes*. Hoeprich (10) suggested penicillin as the drug of choice for the treatment of listeriosis because 0.5 μg/ml was usually bactericidal for *Listeria*. Our MBC results agreed with those of Moellering et al. (15); however, we showed that the percent kill is greatly influenced by the initial inoculum size. Had we used an inoculum of 10^5 and defined MBC as 99% kill, the MBC end points would have been different.

Although bacteriostatic antibiotics have been used with good results for the treatment of listeriosis (3, 4, 20), bactericidal antibiotics have a potential advantage for patients with impaired host-defense mechanisms (15) and for neonates, who, in many respects, are deficient hosts (13).

Several combinations of antibiotics have been reported to have a synergistic effect on some strains of *Listeria*. These include penicillin and sulfonamides or streptomycin (20); carbenicillin, penicillin, ampicillin and kanamycin, gentamicin, and streptomycin (8, 13, 15).

For several years we have received information regarding therapy from physicians who have submitted *Listeria* strains isolated from neonates. In almost all cases the initial treatment included penicillin or ampicillin with kanamycin or gentamicin. We evaluated the in vitro synergistic effect of ampicillin and gentamicin and found that this combination gave earlier and more complete killing than did either agent alone. With 40 of 45 strains tested, the CFU per milliliter with the combined antibiotics was decreased by ≥ 2 $\log_{10}$ after 24 h, as compared with killings by either antibiotic alone. Our results support those (8) which suggest that the broad antibacterial combination therapy usually given neonates with bacterial infection even before the causative organism is identified may contribute to recovery from listeriosis. However, the relevance of these in vitro observations to the efficacy of treatment of patients will have to be established in clinical studies.

In summary, a large number of recent clinical isolates of *L. monocytogenes* appear to be homogeneous in their susceptibility to commonly used antibiotics. Our data show that in vitro results are greatly influenced by the methodology used, i.e., inoculum size, media, and the definition used for end points.

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