ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1978, p. 791-795
0066-4804/78/0013-0791$02.00/0

Vol. 13, No. 5
Printed in U.S.A.

Nitrate Reduction: New Method for Testing the Antibiotic Susceptibility of *Haemophilus influenzae*

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Received for publication 10 November 1977

We have developed a new micro-broth-dilution assay for determining the antimicrobial susceptibility of *Haemophilus influenzae*. This assay is based on the ability of viable *H. influenzae* to reduce nitrates to nitrites. Bacterial viability is detected by a positive nitrite reaction rather than visible turbidity. The nitrate reduction assay was compared with a standard microassay using 51 isolates of *H. influenzae* and six beta-lactam antibiotics. Although there was good agreement between the two methods, the nitrate reduction assay was more sensitive in detecting viable bacteria, and so established a more accurate estimate of the minimal inhibitory concentration. The nitrate reduction assay offered the additional advantage that it could be used to determine the minimal bactericidal concentration without having to subculture the broth. Ampicillin, penicillin, and cefamandole were equally effective in vitro against susceptible strains (minimal inhibitory concentrations, 0.125 to 0.5 μg/ml), whereas all three antibiotics were ineffective against two beta-lactamase-producing strains. Using the nitrate reduction assay, resistance to cefamandole was detectable with inoculum sizes ranging from 10^4 to 10^6 colony-forming units per ml, while the turbidity assay detected resistance only with the largest inoculum.

The appearance of ampicillin- and chloramphenicol-resistant strains of *Haemophilus influenzae* (9, 14) has stimulated interest in developing better techniques for assaying the antimicrobial susceptibility of these fastidious bacteria (5, 6). Broth dilution assays, though commonly employed to measure both the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of antimicrobial agents, can be used to test the susceptibility of *H. influenzae* only if the medium is supplemented with hemin and nicotinamide adenine dinucleotide. Some broths will support the growth of *H. influenzae*, but even under good growth conditions turbidity may be difficult to detect (4, 7).

We have developed a new assay procedure for testing the antimicrobial susceptibility of *H. influenzae*. The assay is based on the nitrate reductase activity of viable *H. influenzae* and the resultant production of nitrite (4, 16). Our method has the distinct advantage of increased reproducibility and accuracy, since the MIC is indicated by a color reaction, which is more easily detected than turbidity. This report includes a description of the assay and the results obtained with several beta-lactam antibiotics.

MATERIALS AND METHODS

Bacteria. Fifty-one isolates of *H. influenzae* were tested. Thirty-nine isolates were smooth, nontypable *H. influenzae* recovered from sputum specimens submitted to the bacteriology laboratory, San Diego Veterans Administration Hospital. Ten isolates from various clinical specimens were classified serologically as type B and were ampicillin susceptible. Two strains were ampicillin-resistant, beta-lactamase-producing type B strains; one was obtained from Clyde Thornsberry, Center for Disease Control, Atlanta, Ga., and the other from Charles Davis, University Hospital, San Diego, Calif. All isolates were identified as *H. influenzae* by demonstrating a requirement for both X and V factors. Nitrate reductase activity was demonstrated by a modification of the Cook method (1), using 5% defibrinated sheep blood, 0.2% KNO_3, and 5% Fildes digest (Difco) in the agar. We tested all isolates for beta-lactamase activity with the phenol-red indicator method of Rosen et al. (13). All cultures were stored at −70°C in defibrinated sheep blood until they were used (11).

Antibiotic susceptibility tests. Supplemented brucella broth (SBB), consisting of brucella broth (Pfizer) supplemented with 3% Fildes digest and 0.1% KNO_3, was used both as the growth medium and as the diluent for the antibiotics. Wells of plastic microdilution trays (Cooke Engineering Co., Alexandria, Va.) were filled with 0.1 ml of SBB containing serial

twofold dilutions of the antibiotics. The inoculum was 0.1 ml of an overnight SBB culture diluted to contain 10^5 colony-forming units (CFU) per ml. The population densities of the inocula were confirmed by colony counts. The microdilution trays were incubated at 35°C without added carbon dioxide. After 16 to 18 h of incubation, trays were examined for visible turbidity to determine MIC, and those wells without visible growth were subcultured to Fildes enrichment agar with a 0.01-ml calibrated loop to determine MBC. The nitrate test (12) was then performed by adding to each well, with a tuberculin syringe and a 27-gauge needle, 1 drop of sulfanilic acid (0.8% in 5 N acetic acid) followed by 1 drop of α-naphthylamine (0.5% in 5 N acetic acid). A bright red color, which appeared within 5 min of adding the reagents, was interpreted as a positive test for nitrite. Each assay was performed in duplicate.

Antimicrobial agents. The seven antimicrobial agents tested and their suppliers were: ampicillin, Bristol Laboratories, Syracuse, N.Y.; tetracycline and penicillin G, Nutritional Biochemicals Corp., Cleveland, Ohio; cephalothin, cefamandole, cephalexin, and cefazolin, Eli Lilly, Indianapolis, Ind. All antibiotics were stored in desiccators at 4°C. Aqueous solutions were freshly prepared immediately before each test and contained 1,000 μg of active antibiotic per ml.

Beta-lactamase activity. Bacteria were grown overnight in SBB, then diluted to provide an inoculum of 10^5 CFU/ml in 50 ml of fresh SBB containing 8 μg of cefamandole per ml. The bacteria were incubated in the antibiotic medium at 35°C for 24 h. A 6-ml sample was removed from each flask at 0, 3, 6, 9, 12, and 24 h. Five milliliters of the sample was centrifuged, filtered through a 0.45-μm membrane filter, and then heated at 85°C for 30 s to inactivate beta-lactamase before being assayed for residual antibiotic. The size of the bacterial population was determined using the remaining 1.0 ml. A control flask without bacteria was treated in the same manner.

Concentrations of cefamandole were measured using antibiotic medium no. 5 (BBL) with *Bacillus subtilis* ATCC-6633 as the indicator organism. Standards were prepared in SBB at the beginning of the experiment and stored frozen at −70°C until used. Twenty microliters of each sample described above and of the standards were applied to 6-mm paper disks, which were then placed on the agar surface. After overnight incubation, diameters of the growth-free zones were measured and the mean was calculated. Each assay was run in quadruplicate.

RESULTS

The MIC of each antibiotic was determined in duplicate for 51 strains of *H. influenzae*. When visible turbidity was used to estimate the MIC, 25% of the duplicate assay results varied by one dilution. (The higher concentration of antibiotic was accepted as the MIC in those instances). The results of duplicate tests using the nitrate reduction assay (NRA) were always in agreement.

Table 1 compares the MIC obtained by visual inspection with that obtained by NRA. The results corresponded for 79% of the tests. In the remaining comparisons, the MIC determined by the NRA was one or two dilutions higher. Those wells that appeared to be clear, but had positive nitrite reactions, were found to contain between 10^4 and 10^5 CFU/ml. Wells with a negative nitrite reaction were sterile upon subculture to agar. A discrepancy between the two methods was found more often with the less effective cephalosporins than with the three more potent antibiotics (53/153 versus 8/153).

Both assays clearly differentiated between the beta-lactamase-producing strains and the ampicillin-susceptible strains. Both assays also differentiated the beta-lactamase-producing strains from cefamandole-susceptible strains, though the MICs of cefamandole were lower than those for ampicillin against the resistant strains (Table 1). The degree of cefamandole resistance was underestimated by the turbidity method. Nonturbid wells with positive nitrite reactions contained 10^4 to 10^5 CFU/ml.

The MIC and MBC of cefamandole were

TABLE 1. *MICs of beta-lactam antibiotics for inhibition of 51 strains of H. influenzae*

Antibiotic	Assay	No. of strains inhibited by MIC (μg/ml)[a]:									
		0.12	0.25	0.5	1	2	4	8	16	32	64
Ampicillin	Turbidity		48	1						2[b]	
	NRA		43	6						2[b]	
Penicillin G	Turbidity	3	46							2[b]	
	NRA	2	47							2[b]	
Cefamandole	Turbidity	2	46	1			1[b]	1[b]			
	NRA	2	45	2					2[b]		
Cephalothin	Turbidity		1	23	27[b]						
	NRA			8	40[b]	3					
Cefazolin	Turbidity			9	35	6[b]	1				
	NRA				13	28[b]	4	6			
Cephalexin	Turbidity						2[b]	45	3	1	
	NRA						1	34[b]	15	1	

[a] Inoculum was 10^5 CFU/ml.

[b] Includes β-lactamase-producing strains.

markedly affected by the size of the inoculum of beta-lactamase-producing strains. It is noteworthy that, with all three inocula, the NRA MIC was higher than that estimated by turbidity. Neither the inoculum effect nor the discrepancy between the NRA and turbidity results was observed with cefamandole-susceptible strains (Table 2).

Two strains of *H. influenzae* with beta-lactamase activity were tested for their ability to inactivate cefamandole. The concentration of cefamandole decreased until it fell below the MIC, after which bacterial regrowth occurred (Fig. 1). Ampicillin was inactivated more rapidly by these strains; no detectable ampicillin remained after 2 h of incubation with these bacteria (data not shown).

Susceptibility to cefamandole of the beta-lactamase-producing strains of *H. influenzae* was measured before and after exposure to cefamandole. The MIC did not change, providing further evidence that cefamandole resistance was not due to the presence in the original inoculum of a small number of highly resistant mutants.

In most of this work, the NRA was used only to measure the MIC of penicillins and cephalosporins. We believe, however, that the assay can be used to measure both the MIC and the MBC when they are not equal. We observed that, when the susceptibility of the beta-lactamase-producing *H. influenzae* was tested, there was a four- to eightfold difference between the MIC and the MBC (Table 2). With these organisms, the MIC was, as usual, equivalent to the lowest concentration of antibiotic that resulted in a negative nitrite reaction. However, as the trays were allowed to stand, a pink color developed in one or more wells which initially had been colorless. This pink color represented a weakly positive nitrite reaction and was due to the presence of small numbers (10^3 to 10^4) of surviving bacteria. The lowest concentration of antibiotic that inhibited all pigment production after 30 min of incubation was the MBC. This was confirmed by subculturing all the wells without visible growth with a 0.01-ml loop prior to adding the nitrite reagents.

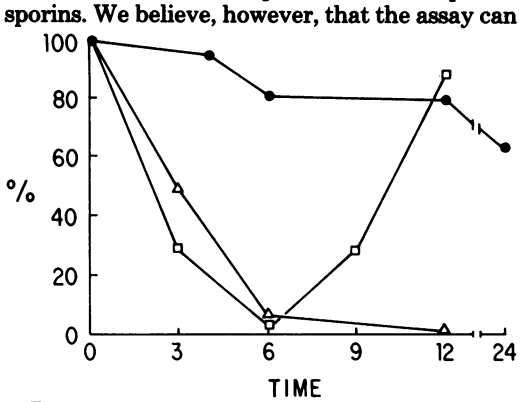

FIG. 1. *A type B, beta-lactamase-producing strain of H. influenzae was added to SBB containing 8 μg of cefamandole per ml so that the bacterial concentration was 5 × 10^5 CFU/ml. (●) Percentage of cefamandole remaining in uninoculated SBB; (△) percentage of cefamandole in broth inoculated with H. influenzae; (□) percentage of the original bacterial inoculum at each interval. Each point is the mean of two assays.*

To verify the ability of the NRA to reliably establish the MBC, we measured the MIC and MBC of tetracycline with 10 strains of *H. influenzae*. The antibiotic characteristically must be present in much higher concentrations to kill bacteria than to inhibit bacterial growth. As expected, the MBC of tetracycline was four to eight times higher than its MIC with all three inoculum sizes (Table 3). The MBC determined by the NRA was identical to the MBC as determined by subculture with each of the 10 strains.

DISCUSSION

In this study we compared a new method for testing the antibiotic susceptibility of *H. influenzae* (NRA) with a standard micro-broth-di-

TABLE 3. *MIC and MBC of tetracycline measured by NRA against H. influenzae*[a]

Inoculum (CFU/ml)	Results (μg/ml) of assay:		
	NRA MIC[b]	MBC[c]	NRA MBC[d]
10^4	0.25	1	1
10^5	0.25	2	2
10^6	0.25	2	2

[a] Ten strains tested.
[b] Immediate color reaction.
[c] Based on subculture.
[d] After 30 min of incubation.

TABLE 2. *Effect of increasing inoculum size on susceptibility of H. influenzae to cefamandole*

Inoculum (CFU/ml)	β-Lactamase negative (10 strains)			β-Lactamase positive (2 strains)		
	MIC (μg/ml)		MBC (μg/ml)[a]	MIC (μg/ml)		MBC (μg/ml)[a]
	Turbidity	NRA		Turbidity	NRA	
10^4	0.12	0.12	0.12	1.0	4	16
10^5	0.25	0.25	0.25	4.0	16	64
10^6	0.25	0.25	0.25	4.0	32	>64

[a] Based on subculture to agar.

lution technique. The comparison was made by determining the MIC of six beta-lactam antibiotics for 51 isolates of *H. influenzae*, including two beta-lactamase-producing strains. With the standard assay technique, the lowest concentration of antibiotic to inhibit the development of visible turbidity was defined as the end point, or the MIC. The end point for the NRA was the lowest concentration of antibiotic to prevent the reduction of nitrates to nitrites as demonstrated by a qualitative nitrite test, i.e., the appearance of a red color after addition of reagents.

The MICs were identical with both methods in 240 of 306 comparisons. When results were disparate, the MIC determined by the NRA was always higher. This was due to the greater sensitivity of the NRA in detecting bacterial growth. Visible turbidity could be detected only when the bacterial density was $>10^6$ CFU/ml, while a density of $\geq 10^5$ was sufficient to give a positive nitrite reaction. Since the original inoculum was 10^5 CFU/ml, we believe that the results obtained with NRA more closely approximate a true MIC. The sensitivity of the nitrite test can be increased, if desired, by increasing the substrate concentration or working with large volumes. We chose our conditions so that the threshold for a positive test would approximate the original inoculum.

When determined by the NRA, the MICs of penicillin G and ampicillin for the 49 susceptible strains were nearly identical, as reported by McLenn et al. (8). The effective range of ampicillin (0.125 to 0.5 μg/ml) is similar to previously reported values determined by broth dilution (5) and agar dilution techniques (3, 15), further supporting the validity of the NRA.

There was wide variation in the in vitro efficacy of the four cephalosporins tested. Cefamandole was the only cephalosporin that was as effective as the penicillins. The MICs of the other three cephalosporins were from 4 to 50 times greater than that of ampicillin. Beta-lactamase production did not seem to be an important determinant of susceptibility to cephalothin, cefazolin, or cephalexin. It has been reported that cephalothin is a poor substrate for this enzyme (6). However, beta-lactamase-producing strains were resistant to cefamandole, and this antibiotic was hydrolyzed by the bacteria (Fig. 1).

Kattan et al. made the paradoxical observation that beta-lactamase-producing *H. influenzae* were susceptible in vitro to cefamandole even though cefamandole was hydrolyzed by beta-lactamase from *H. influenzae* nearly as rapidly as was benzylpenicillin (6). We found that beta-lactamase-producing strains were resistant to cefamandole. This difference in results might be explained by the difference in inoculum sizes; we used 10^5 CFU/ml, and Kattan et al. used only 10^3 CFU/ml. When we tested resistant *H. influenzae*, increasing the inoculum size increased the MIC of cefamandole (Table 2) in the same manner that inoculum size influenced ampicillin susceptibility (10). When the inoculum (and therefore the amount of beta-lactamase) was small, resistant *H. influenzae* appeared to be susceptible to ampicillin and/or cefamandole. This may have been due, in part, to the lack of a permeability barrier to ampicillin (and cefamandole). These antibiotics can penetrate the cell and inhibit bacterial growth if the antibiotics are not first hydrolyzed by the beta-lactamase (10). Clinical experience has shown that ampicillin is often ineffective therapy for meningitis due to beta-lactamase-producing strains. Whether this will be true for cefamandole is not known, but our results suggest caution before recommending cefamandole as an alternative therapy for beta-lactamase-producing strains (2).

The NRA may be useful for the rapid determination of MBC as well as MIC. We found that MBC could be determined after incubating microdilution trays for 30 min at room temperature, at which time there was a weakly positive nitrite reaction, as evidenced by the development of a pink color, in those wells with between 10^3 and 10^5 CFU/ml. Those wells with a completely negative nitrite reaction, even after prolonged incubation, had no growth on subculture. This modification was used successfully to determine the MBC of tetracycline. We believe that the NRA will probably be useful for determining the MIC and MBC of most antimicrobial agents.

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