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Rapid Detection of Ampicillin-Resistant *Haemophilus influenzae* and Their Susceptibility to Sixteen Antibiotics

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Ampicillin-resistant and -susceptible strains of *Haemophilus influenzae* were tested for susceptibility to 16 antibiotics. Chloramphenicol and a new cephalosporin, cefamandole, were most active with minimal inhibitory concentrations (MICs) for all bacteria tested between 0.5 to 2.0 μg/ml. All but two organisms were susceptible to tetracycline. Ampicillin-resistant strains of *H. influenzae* were less susceptible (MIC, 4 to 32 μg/ml) to carbenicillin and ticarcillin than ampicillin-susceptible organisms (MIC, 0.25 to 1.0 μg/ml). A rapid assay for β-lactamase, utilizing a chromogenic cephalosporin substrate, detected enzyme production in all 17 ampicillin-resistant strains of *H. influenzae*.

Several investigators have recently reported the isolation of *Haemophilus influenzae* strains resistant to ampicillin (1, 2, 5, 10, 13-17). The majority of these were clinical isolates from meningitis patients responding poorly to ampicillin therapy. In most cases, these patients have responded to subsequent chloramphenicol therapy, although two died. It is now extremely important for clinicians to anticipate the possibility of ampicillin resistance in *H. influenzae* and to have available prompt and precise means of detecting these resistant strains. Determination of antibiotic susceptibility usually requires 48 h from the time a clinical specimen is collected. However, one characteristic that uniquely distinguishes an ampicillin-resistant strain of *H. influenzae* is the constitutive elaboration of a β-lactamase (4, 5, 11). Detection of this enzyme at the time of primary isolation would allow initiation of appropriate therapy at least 24 h sooner than normally achieved by antibiotic susceptibility testing.

This report describes a simple, direct method for detecting β-lactamase production by these organisms, thus identifying ampicillin resistance. In addition, 17 ampicillin-resistant and 17 ampicillin-susceptible strains of *H. influenzae* were tested for susceptibility to 16 antibiotics, including seven new cephalosporin compounds.

MATERIALS AND METHODS

Organisms. Thirty-four strains of *H. influenzae* were studied. Seventeen ampicillin-resistant strains were provided by Lynn Harding, Linda Kirven, and Clyde Thornsberry. Seventeen ampicillin-susceptible strains were submitted to the Lilly Research Laboratories by V. M. Howie and S. E. McLinn for antibiotic susceptibility studies. All strains were clinical isolates.

Antibiotics. The bacteria were tested for susceptibility to ampicillin (Bristol Laboratories, Syracuse, N.Y.), carbenicillin (Roerig Pharmaceuticals, New York, N.Y.), chloramphenicol (Parke, Davis and Co., Detroit, Mich.), tetracycline (Lederle Laboratories, Pearl River, N.Y.), ticarcillin (Beecham-Massengill, Bristol, Tenn.), cefoxitin (Merck Sharp & Dohme, West Point, Pa.), cephacetrile (Ciba Pharmaceutical Co., Summit, N.J.), cephapirin (Bristol Laboratories, Syracuse, N.Y.), and cephradine (Squibb & Sons, E. R., Princeton, N.J.). Cephalothin, cephaloridine, cefazolin, cephalexin, cefamandole, compound 110264, and compound 99638 were supplied by Lilly Research Laboratories.

The chromogenic cephalosporin substrate, compound 87/312, was supplied by Glaxo Research Ltd., Greenford, Middlesex, England.

Susceptibility testing. Minimal inhibitory concentrations (MIC) were determined utilizing a modification of the ICS agar dilution method as outlined in the report of an international collaborative study (3). Inocula for agar dilution susceptibility studies were grown in Trypticase soy broth (BBL), containing 5% defibrinated rabbit blood and 1% IsoVitaleX (BBL). After overnight incubation at 37 C in a 10% CO_2 atmosphere, the cultures were diluted 1:100 in sterile physiological saline. Chocolate agar was prepared by addition of 5% defibrinated rabbit blood and 1% IsoVitaleX (BBL) to Mueller-Hinton medium and held at 60 C for 5 min.

Agar plates, containing the drugs in $\log_2$ concentration, were spot inoculated with a replicating device similar to the Steers replicator (9). Inoculated plates were allowed to air dry for approximately 30 min and then placed in an incubator containing a 10% CO_2 atmosphere. MIC values were determined after overnight incubation at 37 C.

β-Lactamase determination. Use of the chromogenic cephalosporin substrate (Glaxo compound $^{87}/_{312}$)

to detect β-lactamases was originally described by O'Callaghan et al. (8). This compound was shown to act as a substrate for many β-lactamases, from both gram-positive and -negative organisms. Upon hydrolysis of the lactam bond in this cephalosporin, the maximum absorption changes from 386 to 482 nm, that is, from yellow to red.

Bacterial cell suspensions were prepared by picking several colonies of the test organism from a fresh overnight chocolate agar culture and emulsifying these in sterile physiological saline (0.5 ml). Aliquots (40 μl) of the cell suspensions were placed into the wells of a spot plate (model 96U-WS; Linbro Chemical Co., Inc., New Haven, Conn.) containing 50 μl of an aqueous solution (0.5 mg/ml in 0.05 M KPO_4, pH 7.0) of the chromogenic substrate (Glaxo compound $^{87}/_{312}$) at room temperature. A color change from yellow to red occurs almost immediately in wells containing suspensions of β-lactamase-producing organisms. Wells containing no bacteria or non-β-lactamase-producing organisms remain yellow indefinitely.

RESULTS

β-Lactamase detection. Addition of cell suspensions from all 17 ampicillin-resistant strains (MIC, 16 to 128 μg/ml) of *H. influenzae* to the chromogenic cephalosporin substrate resulted in an immediate color change from yellow to red (see Fig. 1, left 17 wells). With 17 ampicillin-susceptible strains (MIC, 0.25 to 1 μg/ml) the color remained the original yellow (see Fig. 1, right 17 wells). Identical results were obtained when these same strains were tested for β-lactamase production using an acidometric method similar to theat described by Thornsberry and Kirven (11). Both methods showed that β-lactamase is produced by the ampicillin-resistant organisms but not by the ampicillin-susceptible strains.

Susceptibility studies. Agar dilution MICs of 16 antibiotics for 34 strains of *H. influenzae* are shown in Table 1. Seventeen ampicillin-susceptible strains with MICs of 0.25 to 1.0 μg/ml are clearly separated in this test from the ampicillin-resistant organisms (MICs, 16 to 128 μg/ml).

Carbenicillin and ticarcillin susceptibilities reveal an identical separation of strains, although the spread between the two groups is not as broad as with ampicillin. Antibiotic susceptibility of these 34 strains to the other antibiotics shows relatively well-defined single groups. One strain (W-Best) stands out as very susceptible to all of the antibiotics. The most active of the non-penicillin compounds were chloramphenicol and cefamandole, with an MIC range for both being 0.5 to 2 μg/ml.

Among the other antibiotics tested, tetracycline and compound 110264 show the widest divergence of MIC values, with ranges of (except for strain W-Best) 1.0 to 128 μg/ml and 2 to 16 μg/ml, respectively.

Ampicillin resistance of these bacteria is not reflected in their response to cephalosporins or chloramphenicol. Two of the ampicillin-resistant strains were, however, also resistant to tetracycline, with MICs of 128 μg/ml.

DISCUSSION

Because strains of *H. influenzae* resistant to ampicillin are now being encountered in clinical

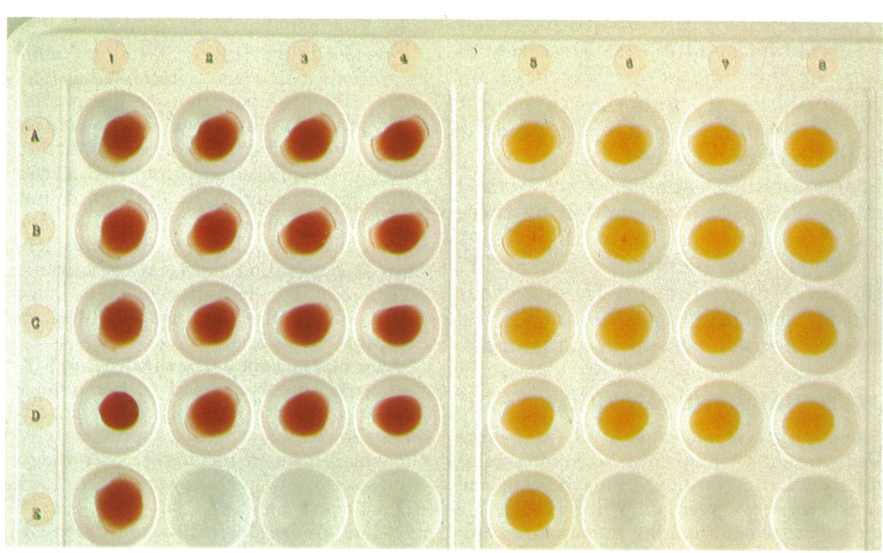

FIG. 1. *Spot plate test utilizing the chromogenic cephalosporin substrate for detection of β-lactamase production by H. influenzae.*

TABLE 1. *Distribution of agar dilution MICs for 34 strains of H. influenzae*

Antibiotic	MIC (μg/ml)										
	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32	64	128
Ampicillin		3[a]	13	1				2	6	6	3
Carbenicillin		9	6	2			10	1	6		
Ticarcillin		12	4	1		2	1	9	5		
Chloramphenicol			11	22	1						
Cefamandole			5	20	9						
Cefoxitin					3	30	1				
Cephalothin				1[b]	1	12	20				
Cephapirin	1[b]				4	20	9				
99638					5	24	5				
110264			1[b]		5	13	12	3			
Tetracycline				3	18	10			1		2
Cephaloridine				1[b]			12	5	16		
Cefazolin			1[b]				2	30	1		
Cephacetrile				1[b]					11	22	
Cephalexin								1	24	9	
Cephradine			1[b]					1	7	16	9

[a] Number of strains.
[b] Strain W-Best.

medicine, reliable methods of detection and safe therapeutic alternatives are necessary. Susceptibility studies must now be regularly performed on all clinical isolates, as recently emphasized by Thornsberry and Kirven (12). The use of tests to detect β-lactamase production can save valuable time (11).

The use of the chromogenic substrate assay described herein has several advantages over either the phenol red assay or the iodine starch assay. Buffer and pH control are not as critical as in the phenol red assay. There is no nonspecific reaction like that often observed with cephalosporins in the iodine starch assay (17). In addition, the chromogenic substrate assay is simple and very rapid. We have encountered neither false-positive nor false-negative results with this test when used to detect β-lactamase production. Without exception, strains of *H. influenzae* deemed resistant to ampicillin by susceptibility tests did produce a β-lactamase. Application of the chromogenic substrate method to body fluids, particularly cerebrospinal fluid, should be studied.

The therapeutic dilemma faced by clinicians treating patients with serious *Haemophilus* infections is by no means resolved. The use of ampicillin as the sole therapeutic agent before the results of susceptibility studies are available demands intense clinical vigilance. Nelson (6) has recently stressed the importance of repeating the lumbar puncture with cerebrospinal fluid smears and cultures plus blood cultures on the day after starting therapy, if ampicillin is used alone. Two deaths have occurred during ampicillin therapy for *Haemophilus* meningitis due to resistant strains (12).

Chloramphenicol is reliably active against all strains of *H. influenzae* and has been used successfully in the treatment of meningitis. Potential hematologic toxicity makes it a less than ideal agent. Carbenicillin, ticarcillin, and tetracycline are all active in vitro against most strains of *H. influenzae*, but several isolates were inhibited only at 32 μg/ml or greater. Those organisms resistant to ampicillin were least susceptible to carbenicillin and ticarcillin. This may indicate some instability of these compounds to the *Haemophilus* β-lactamases.

The cephalosporin antibiotics have not been widely used in the treatment of serious infections due to *H. influenzae*, especially meningitis. This reflected the availability of a more active drug with better penetration into the cerebrospinal fluid.

Among the newer cephalosporins only cefamandole has in vitro activity comparable to chloramphenicol. In contrast to carbenicillin and ticarcillin, cefamandole is equally active against ampicillin-susceptible and -resistant *Haemophilus*. Whether adequate concentrations will enter the cerebrospinal fluid to translate these attributes into clinical efficacy is yet to be determined. Cefoxitin is somewhat less

active than cefamandole but, like cefamandole, is resistant to hydrolysis by the *Haemophilus* β-lactamases.

Of the orally active cephalosporins tested, compounds 110264 and 99638 were most active. These may prove useful for treating less serious *Haemophilus* infections, i.e., otitis media.

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