Effect of Inoculum Size and \( \beta \)-Lactamase Production on In Vitro Activity of New Cephalosporins Against Haemophilus Species

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Sixty-three strains of Haemophilus species, 38 of which were \( \beta \)-lactamase producers (37 H. influenzae type b, 1 H. parainfluenzae) and 25 of which were \( \beta \)-lactamase negative (20 H. influenzae, 5 H. parainfluenzae), were tested for susceptibility to cefoxitin, moxalactam (LY127935) (Lilly), cefosulodin (CGP 7174 E, Ciba), and cefoperazone (T 1551, Pfizer). Cefosulodin was relatively inactive at both low and high inocula. LY127935 and cefoperazone displayed inoculum-dependent bactericidal activity. Cefoxitin displayed little inoculum effect against \( \beta \)-lactamase-producing strains: 8 and 16 \( \mu \)g/ml killed at least 90\% of those tested at \( 10^4 \) and \( 10^8 \) colony-forming units per ml, respectively.

It has been well established that inoculum size substantially affects the susceptibility of Haemophilus influenzae to all of the currently commercially available cephalosporins (6), as well as cefamandole (5, 10,18, 24), cefoxitin (5), and cefaclor (2, 23). The purpose of this investigation was to examine the effects of inoculum size on the inhibitory and bactericidal activities of cefoxitin, moxalactam (LY127935), cefoperazone, and cefosulodin against \( \beta \)-lactamase-positive and -negative strains of Haemophilus.

MATERIALS AND METHODS

Organisms. Most of the 63 organisms studied were recent isolates from the Clinical Microbiology laboratories of the Mayo Clinic, Rochester, Minn. Twelve strains of H. influenzae type b were obtained from the Center for Disease Control, Atlanta, Ga., and another 16 were frozen reference strains of \( \beta \)-lactamase-positive Haemophilus. The sources of the isolates and their respective numbers were as follows: sterile body fluids (blood, cerebrospinal fluid), 31; corynopharynx, 13; eye, 2; sputum, 8; and source unknown, 9.

Of the 38 \( \beta \)-lactamase-positive strains, 37 were H. influenzae type b and one was H. parainfluenzae. Of the 25 \( \beta \)-lactamase-negative strains, 12 were H. influenzae type b, 8 were non-typeable H. influenzae, and 5 were H. parainfluenzae. Identification of strains was based on the taxonomic system described by Kilian (11). Serological typing was performed by the immuno-fluorescent-antibody technique. \( \beta \)-Lactamase activity was determined by a rapid slide technique (16). Reference strains of Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923, and Pseudomonas aeruginosa Mayo strain were used as controls when determining minimum inhibitory concentrations (MICs). All organisms were subcultured to solid media to check for purity before inoculation into broth. Isolates were stored in rabbit blood at \(-70^\circ\)C.

Antimicrobial agents. The following antimicrobial agents were used: cefoxitin (Merck, Sharp & Dohme, West Point, Pa.); cefosulodin, or CGP 7174 E (Ciba Pharmaceutical Co., Summit, N.J.); cefoperazone, or T 1551 (Pfizer Pharmaceuticals, New York, N.Y.); and moxalactam (LY127935; Lilly Research Laboratories, Indianapolis, Ind.). Antibiotics were solubilized in appropriate diluents (21), and stock solutions were either used immediately or stored frozen at \(-40^\circ\)C.

Media. The following media were briefly evaluated for their suitability in this study: (i) Mueller-Hinton broth (MHB) supplemented with 10 \( \mu \)g of hemin and 10 \( \mu \)g of ninocaminidine adenine dinucleotide (NAD) per ml (4); (ii) Schaedler broth supplemented with 5\% Fildes extract (peptic digest of blood) (19); and (iii) MHB supplemented with 5\% lyzed horse blood and 2.5 \( \mu \)g of NAD per ml (12). Such an evaluation was deemed appropriate, because some investigators have encountered problems with poor growth and reproducibility of susceptibility studies with Haemophilus species (1, 3, 8, 9, 22).

The three media were evaluated by testing the susceptibility of a \( \beta \)-lactamase-positive and a \( \beta \)-lactamase-negative strain of H. influenzae to ampicillin and cefamandole in each medium. The specific criteria examined were growth characteristics, ease of interpretation of endpoints in both macro- and microdilution systems, and reproducibility of endpoints.

Susceptibility test. MICs were determined by a broth microdilution method. Antimicrobial dilutions were dispensed into plates with the MIC 2000 dispenser (Dyntech, Inc., Alexandria, Va.) and stored at \(-70^\circ\)C. Isolates of Haemophilus were subcultured to fresh chocolate agar plates, grown overnight and then subcultured into two tubes containing 2 and 20 ml of Schaedler broth, respectively. The light inoculum was prepared by adjusting the turbidity of the inoculum in broth to match that of one-half of a McFarland barium sulfate standard (approximately \( 10^8 \) colony-forming units [CFU] per ml) and then diluting the adjusted inoculum 1:10 (21). The heavy inoculum was prepared by adjusting the turbidity of the inoculum in broth to match that of a no. 3 McFarland barium sulfate standard (approximately \( 9 \times 10^8 \) CFU/ml). The two ad-
justed organism suspensions were then delivered into the microdilution trays with the MIC 2000 inoculator so that the final light and heavy inocula were 10⁶ and 10⁷ CFU/ml, respectively. Triplicate plate counts were performed to ensure that the actual colony counts correlated well with those predicted by adjusting turbidity. The microdilution plates were incubated for 24 h at 35°C in sealed bags, without CO₂. The MIC was defined as that concentration in which there was no visible growth; a slight opacity, shown to be due to spheroplast formation at the higher inoculum, was disregarded (13). Minimum bactericidal concentrations (MBCs) were determined by delivering 50-µl samples of broth from the last turbid well and the next five wells without visible growth onto a chocolate agar plate. These plates were incubated at 35°C for 6 h in 5% CO₂. The MBC was the least concentration of antibiotic which yielded no growth upon subculture.

RESULTS

All three media evaluated readily supported growth of H. influenzae type b and gave similar and reproducible MICs for ampicillin and cephalosporin. Schaedler broth with Fildes extract and MHB with NAD and hemin provided clearer endpoints than those obtained in MHB with lysed horse blood and NAD. MHB with NAD and hemin, however, failed to yield adequate growth in the microdilution system of 30 strains of unencapsulated and nontypable strains of H. influenzae. For these reasons and because of its ease of preparation, Schaedler broth with Fildes extract was used during the remainder of the study.

The results of testing the 63 strains of Haemophilus are shown in the accompanying tables. The cumulative percentage of 25 strains of β-lactamase-negative strains of Haemophilus inhibited (MIC) and killed (MBC) for each antimicrobial agent at two different inoculum sizes is shown in Table 1. At the generally recommended inoculum, 10⁶ CFU/ml (20), cefusulodin was relatively inactive; cefoperazone and LY127935 were the most active, followed closely by cefoxitin. LY127935 and cefoperazone displayed inoculum-dependent bactericidal effects; however, even against an inoculum of 10⁶ CFU/ml, both of these compounds killed >80% of strains at 2 µg/ml and >90% at 8 µg/ml. Cefoxitin demonstrated no appreciable inoculum effect and was bactericidal to all β-lactamase-negative strains at a concentration of 16 µg/ml.

The effects of inoculum size and β-lactamase production on the susceptibility of 38 strains of Haemophilus are shown in Table 2. At an inoculum of 10⁶ CFU/ml, LY127935 and cefoperazone were the most inhibitory and bactericidal of the compounds tested. Both compounds displayed substantial inoculum-dependent effects on both inhibitory and bactericidal activity;
however, LY127935 was bactericidal at a concentration of 8 μg/ml against all but one strain at the higher inoculum. Comparable bactericidal activity but without inoculum effect was displayed by cefoxitin.

**DISCUSSION**

Under all experimental conditions, LY127935 was the most inhibitory and bactericidal compound against all 63 strains of *Haemophilus*. Cefoperazone had similar activity under standard conditions, but showed marked inoculum-dependent decreases in inhibitory and bactericidal activity when tested against β-lactamase-producing organisms. Although somewhat less active at low concentrations with standard inocula than LY127935 and cefoperazone, cefoxitin was unaffected by high inocula of β-lactamase-producing strains of *Haemophilus*, corroborating data previously published by Eickhoff and Ehret (5).

In their evaluations of LY127935 and of cefoperazone, Neu et al. (14, 15) reported that 90% of *H. influenzae* tested at 10³ CFU/ml were inhibited by 1.6 and 0.8 μg of these compounds per ml, respectively. It is not clear in either study how many β-lactamase-producing strains were tested. Moreover, they performed no tests of the bactericidal activity of the two compounds against *Haemophilus*.

The inoculum-dependent formation of spheroplasts (3, 13, 17) and the problems posed by the medium (1, 4, 8, 9, 19, 22) make it difficult to arrive at uniformly agreed upon endpoints and at a suitable definition for susceptibility of *Haemophilus* to β-lactam antibiotics; however, the known numbers of bacteria in the cerebrospinal fluid of patients with *H. influenzae* meningitis (7) suggest the unrealistic nature and doubtful clinical significance of determining the inhibitory activity of β-lactam antibiotics against 10⁴ CFU of *H. influenzae* per ml. The purported activity of cephalosporins against *Haemophilus* must, therefore, be interpreted cautiously unless results include the inhibitory and bactericidal activities of such compounds against high inocula of organisms.

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**LITERATURE CITED**


