

## In Vitro Susceptibility of *Haemophilus influenzae* to Sulfamethoxazole-Trimethoprim and Cefaclor, Cephalexin, and Cephadrine

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Sulfamethoxazole-trimethoprim and three oral cephalosporins, cefaclor, cephalexin, and cephradine, were evaluated in vitro as possible alternatives to chloramphenicol in the treatment of non-central nervous system infections due to ampicillin-resistant *Haemophilus influenzae*. Sixty-four isolates of *H. influenzae*, including 31  $\beta$ -lactamase-positive strains, were tested by the agar dilution method. All strains were inhibited by 0.78/0.039  $\mu$ g sulfamethoxazole-trimethoprim per ml and by 0.78  $\mu$ g of chloramphenicol per ml. At 6.25  $\mu$ g/ml, 100, 11, and 3% of all strains were inhibited by cefaclor, cephalexin, and cephradine, respectively. Thus, on the basis of drug concentrations presumably achievable in serum, 100% of strains were susceptible to sulfamethoxazole-trimethoprim, chloramphenicol, and cefaclor. However, a considerable inoculum effect was noted with both  $\beta$ -lactamase-positive and -negative strains, when tested with sulfamethoxazole-trimethoprim; the minimal inhibitory concentrations of cefaclor were only slightly affected. Also, synergistic effects of sulfamethoxazole-trimethoprim, sulfamethoxazole-erythromycin, and sulfamethoxazole-cefacylor were seen when combinations were tested against both  $\beta$ -lactamase-positive and -negative strains, as determined by minimal inhibitory concentrations measured by the broth dilution method and by killing curve analyses. These results support further evaluation of these combinations and of cefaclor alone for the treatment of non-central nervous system infections due to *H. influenzae*.

The emergence of *Haemophilus influenzae* strains resistant to ampicillin has created a therapeutic problem. Although chloramphenicol is at present the drug of choice for the treatment of serious infections caused by  $\beta$ -lactamase-positive *H. influenzae*, its toxicity makes it a less than optimal antibiotic for conditions amenable to oral therapy, such as otitis media, cellulitis, bronchitis, bronchopneumonia, and possibly even septic arthritis. No single agent is available for these indications at present, although combinations such as erythromycin and sulfonamide or trimethoprim (TMP) and sulfamethoxazole (SMZ) appear useful by virtue of clinical experience and in vitro activity.

Ampicillin resistance of the majority of *H. influenzae* strains is due to the production of  $\beta$ -lactamase. Some antibiotics of the cephalosporin class are potentially useful because of their resistance to this enzyme, including some newly developed orally absorbable cephalosporins, such as cephalexin, cephradine, and cefaclor. The present study was undertaken to investigate

several drugs singly and in combination as possible alternatives to chloramphenicol for the treatment of non-central nervous system infections due to ampicillin-resistant *H. influenzae*.

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### MATERIALS AND METHODS

**Organisms.** Thirty-three  $\beta$ -lactamase-negative and 31  $\beta$ -lactamase-positive clinical isolates of *H. influenzae* were studied. The sources of isolation and respective numbers were as follows: blood, 16; cerebrospinal fluid, 10; throat, 7; nasopharynx, 6; eye, 3; ear, 3; nose, 2; endotracheal aspirate, 1; sputum, 1; pleural fluid, 2; source unknown, 13. Of the  $\beta$ -lactamase-positive strains, 20 were type b and 11 were nontypable. Of the  $\beta$ -lactamase-negative strains, 20 were type b, 2 type e, 1 type f, and 10 nontypable. Typing was performed by the slide agglutination technique employing commercial antisera (Hyland).  $\beta$ -Lactamase-producing strains were identified by the starch-iodine method (3).

**Antimicrobial agents.** The following antimicrobial agents were used: ampicillin (Ayerst Laboratories, Montreal, Quebec), cephalexin (Eli Lilly & Co., Toronto, Ontario), cefaclor (Eli Lilly & Co., Indianapolis, Ind.), cephradine (E. R. Squibb and Sons, Ltd., Montreal, Quebec), chloramphenicol and erythromycin (Laboratory Center for Disease Control, (L.C.D.C.), Ottawa, Ontario), SMZ (Hoffmann-LaRoche Ltd., Montreal, Quebec), and TMP (Burroughs-Wellcome and Co., London, England). Stock solutions were prepared from powder and either used immediately or frozen at  $-70^{\circ}\text{C}$ . The ratio of SMZ and TMP when used in combination was 20:1.

**Media.** Mueller-Hinton broth or agar (Difco) was used in all experiments except for susceptibility testing and killing curve studies involving TMP or SMZ, where Diagnostic Sensitivity Test broth or agar (Oxide) was employed. These media were supplemented with 5% Fildes enrichment (Difco) and 1% supplement C (Difco). Thymidine phosphorylase (0.1 unit/ml) (Burroughs-Wellcome) was also added whenever TMP or SMZ was tested. For the microtiter broth dilution technique, media were supplemented as described above but without Fildes enrichment, to obtain a clear broth.

**Preparation of inocula.** Several colonies of a pure culture of each strain were inoculated into 10 ml of broth and incubated at  $35^{\circ}\text{C}$  for 16 to 18 h. The optical density of the overnight culture was determined by a spectrophotometer (Spectronic 20, Bausch and Lomb) at 600 nm, and dilutions were made in broth to contain the desired number of colony-forming units (CFU) per milliliter according to a standard curve relating optical density to CFU per milliliter.

**Agar dilution technique.** Agar media containing serial twofold dilutions of antimicrobial agents were prepared. Inocula containing  $10^6$  CFU/ml were applied to the agar surface with a Steers replicator device which delivers approximately 0.002 ml. The inoculated plates were incubated at  $35^{\circ}\text{C}$  for 18 h in a 5%  $\text{CO}_2$  atmosphere.

**Microtiter broth dilution techniques.** Inocula

were adjusted to contain  $2 \times 10^6$  CFU/ml, and 0.05 ml was added to an equal volume of drug suspension. The plates were incubated at  $35^{\circ}\text{C}$  for 18 h. The lowest concentration of drug with no visible turbidity was used to define the minimal inhibitory concentration (MIC) of ampicillin, chloramphenicol, erythromycin, TMP, and cefaclor, whereas a sudden reduction in growth was used for SMZ MIC determinations.

**Effect of inoculum concentration.** The effect of inoculum size on MIC was tested (i) by the agar dilution technique, with all strains using inocula of  $10^3$ ,  $10^4$ ,  $10^5$ , and  $10^6$  CFU/ml, or (ii) by the microtiter broth dilution technique with one strain each of  $\beta$ -lactamase-positive and -negative strains, using final inocula of  $10^3$ ,  $10^4$ ,  $10^5$ , and  $10^6$  CFU/ml. The effect of inoculum size on the synergism of antimicrobials used in combination was also tested by the microtiter broth dilution technique using the four different inocula described above.

**Tests for synergism.** Synergism was measured by two techniques against one strain each of  $\beta$ -lactamase-positive and -negative strains: (i) by checkerboard microtiter broth dilution and (ii) by killing curves. Synergism in broth was defined isobolographically according to the method of Weinstein et al. (10). For killing curve studies, flasks (250 ml) containing 20 ml of broth were inoculated with  $10^5$  CFU/ml and incubated at  $37^{\circ}\text{C}$  on a rotary shaker (200 rpm). Colony counts were performed at 0, 4, 8, and 24 h. Synergism was defined as a reduction of at least 2 logs in the colony counts achieved with combination antimicrobials over those of the drugs tested singly.

## RESULTS

**In vitro susceptibility.** Figure 1 illustrates the in vitro susceptibility of 64 strains of *H. influenzae* (including 31  $\beta$ -lactamase-producing strains) to five antibiotics used singly and to the antimicrobial combination SMZ-TMP, as determined by the agar dilution technique using an inoculum of  $10^6$  CFU/ml. All strains were in-

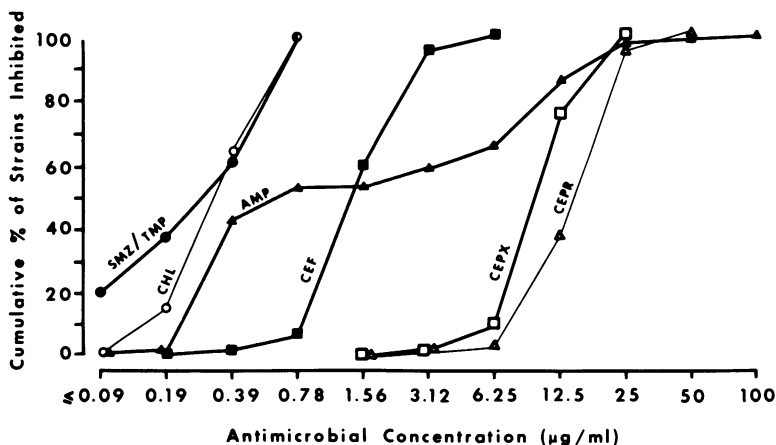


FIG. 1. Agar dilution susceptibilities of 64 *H. influenzae* strains. Inoculum,  $10^6$  CFU/ml. The concentrations of SMZ-TMP are based on those of SMZ. Abbreviations: CHL, chloramphenicol; AMP, ampicillin; CEF, cefaclor; CEPR, cephradine; CEPX, cephalexin.

hibited by 0.78/0.039  $\mu\text{g}$  of SMZ-TMP per ml and by 0.78  $\mu\text{g}$  of chloramphenicol per ml. At 6.25  $\mu\text{g}/\text{ml}$ , 100, 11, and 3% of all strains were inhibited by cefaclor, cephalexin, and cephradine, respectively. With ampicillin, 100% of the  $\beta$ -lactamase-negative strains were inhibited by a concentration of 0.78  $\mu\text{g}/\text{ml}$ ; in contrast,  $\beta$ -lactamase-positive strains exhibited a wide range of susceptibility (i.e., MICs ranged from 3.12 to 100  $\mu\text{g}/\text{ml}$ ) (Table 1).

**Effect of inoculum concentration on MICs.** The effect of increasing the inoculum from  $10^6$  to  $10^7$  CFU/ml was studied with all strains, using the agar dilution technique. A marked effect was noted with SMZ-TMP, with the antimicrobial concentration required to inhibit a given percentage of strains increasing by eightfold. Thus, with an inoculum of  $10^7$  CFU/ml, 6.25/0.312  $\mu\text{g}/\text{ml}$ , instead of 0.78/0.039  $\mu\text{g}/\text{ml}$ , was required to inhibit 100% of the strains. No difference was seen with chloramphenicol, and only a slight effect (a twofold increase in MIC) was seen with cefaclor. As expected,  $\beta$ -lactamase production led to an inoculum effect with ampicillin: MICs of  $\beta$ -lactamase-positive strains increased 2- to 16-fold while those of  $\beta$ -lactamase-negative strains were not affected. The effect of inoculum size on cephalexin and cephradine was not examined (Fig. 1).

This inoculum effect was further investigated using the microtiter broth dilution technique

and inocula of  $10^3$ ,  $10^4$ ,  $10^5$ , and  $10^6$  CFU/ml. For this study, one strain each of  $\beta$ -lactamase-positive and -negative strains were used. No inoculum effect on chloramphenicol and only a minor effect on cefaclor was observed. On the other hand, with an increase from  $10^5$  to  $10^6$  CFU/ml, a marked increase in MIC was noted with SMZ-TMP (1.56/0.078 to 50/2.5  $\mu\text{g}/\text{ml}$ ). With ampicillin, a similar effect occurred with the  $\beta$ -lactamase-positive strain (12.5 to  $>200$   $\mu\text{g}/\text{ml}$ ).

**Synergy studies.** Cefaclor, TMP, and erythromycin were examined for synergy in combination with SMZ by the checkerboard microtiter broth dilution method using an inoculum concentration of  $10^5$  CFU/ml. All three combinations were active, and Fig. 2 illustrates these findings for SMZ-cefclor. Furthermore, varying the inoculum to  $10^3$ ,  $10^4$ , or  $10^6$  CFU/ml did not affect the action of all three combinations (data not shown). The synergistic action of these drugs in combination was also demonstrated by killing curves (Fig. 3) using a  $\beta$ -lactamase-negative strain. Erythromycin and cefaclor were clearly synergistic with SMZ. This effect was also observed with SMZ-TMP (data not shown). Similar results were obtained with a  $\beta$ -lactamase-positive strain.

**DISCUSSION**

This study demonstrates that chloramphenicol and cefaclor when used singly, were the most active agents among the drugs tested in vitro

TABLE 1. Distribution of agar dilution MICs of ampicillin according to  $\beta$ -lactamase production

$\beta$ -Lactamase	No. of strains	Percent of strains <sup>a</sup> inhibited at ( $\mu\text{g}/\text{ml}$ ):										
		$\leq 0.09$	0.19	0.39	0.78	1.56	3.12	6.25	12.5	25	50	100
Negative	33	2.9	2.9	82.4	100	100	100	100	100	100	100	100
Positive	31	0	0	0	0	0	12.9	29	71	93.5	96.8	100

<sup>a</sup> Inoculum,  $10^6$  CFU/ml.

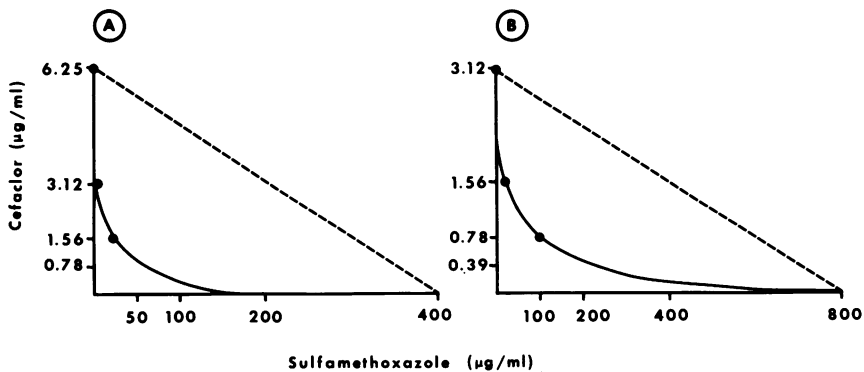


FIG. 2. Isobolograms showing synergism between SMZ and cefaclor. Inocula of  $10^5$  CFU/ml were used. (A)  $\beta$ -Lactamase-positive strain; (B)  $\beta$ -lactamase-negative strain.

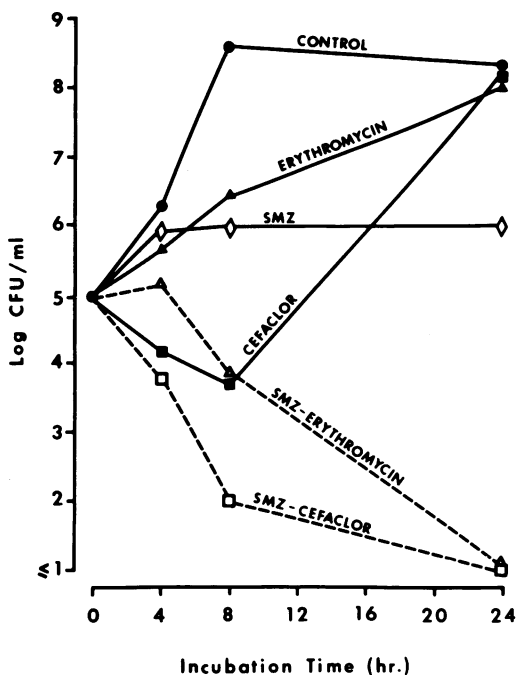


FIG. 3. Rate of killing of a  $\beta$ -lactamase-negative strain by SMZ, erythromycin, and cefaclor alone and in combination. Concentrations of antimicrobials used were: erythromycin, 0.8  $\mu$ g/ml; SMZ, 100  $\mu$ g/ml; cefaclor, 0.8  $\mu$ g/ml.

against all strains of *H. influenzae*. The high degree of activity of SMZ-TMP against both  $\beta$ -lactamase-negative and -positive strains of *H. influenzae* noted in our studies is in agreement with the results of other investigators (7, 8). (In vitro susceptibility of an additional 53 recent clinical isolates of *H. influenzae*, including 27  $\beta$ -lactamase-producing strains, was tested after the present study had been completed. All were susceptible to 1.56  $\mu$ g of SMZ-TMP per ml). However a considerable inoculum effect was observed in both agar dilution and broth dilution studies with this combination.

Certain cephalosporins, by virtue of their relative resistance to hydrolysis by  $\beta$ -lactamase, are potentially useful therapeutic agents against  $\beta$ -lactamase-positive *H. influenzae* infections. However, the results reported here and by others (4, 5, 11) indicate low degrees of activity for cephalexin and cephadrine. Kammer et al. (6), using an inoculum similar to that used in this study (agar dilution,  $10^8$  CFU/ml), showed a better degree of activity for cefoxitin, cephalothin, and cefaclor. Our results with cefaclor agree with theirs. The results reported by Bill and

Washington (1) differed markedly from the above, in that cefaclor inhibited only 10% of  $\beta$ -lactamase-positive and 40% of  $\beta$ -lactamase-negative strains in a similar antibiotic concentration. No explanation could be found for this discrepancy.

Synergy studies confirmed the effect of SMZ on TMP and erythromycin reported by others (2, 9). To the best of our knowledge, the synergism demonstrated between SMZ and cefaclor has not been previously reported. Furthermore, these results were not affected by varying the inoculum from  $10^3$  to  $10^6$  CFU/ml.

#### ACKNOWLEDGMENTS

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