In Vitro Activity of Orally Administered Antimicrobial Agents against *Haemophilus influenzae* Recovered from Children Monitored Longitudinally in a Group Day-Care Center

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To determine whether the prevalence of resistance to commonly used oral antimicrobial agents varied over time, we compared the in vitro susceptibilities of 217 strains of *Haemophilus influenzae* recovered from nasopharyngeal secretions of children in a day-care center studied longitudinally between 1979 and 1987. The overall rate of β -lactamase production in these strains was 18%, with rates of 57% in type b isolates ($n = 21$) and 14% in non-type b isolates ($n = 196$). The percentages of isolates for which MICs were ≤ 1.0 $\mu\text{g/ml}$ for amoxicillin alone, amoxicillin in combination with clavulanic acid, and cefuroxime alone were 82, 92, and 93%, respectively. The percentage of strains for which cefaclor MICs were ≤ 1.0 $\mu\text{g/ml}$ was only 0.5%. Isolates for which chloramphenicol MICs were > 2.0 $\mu\text{g/ml}$ or for which trimethoprim-sulfamethoxazole MICs were $> 0.5/9.5$ $\mu\text{g/ml}$ were uncommon: 1 and $< 1\%$, respectively. High concentrations of erythromycin alone and in combination with sulfisoxazole were required to inhibit the majority of test strains; there was no evidence of erythromycin-sulfisoxazole synergy. In vitro susceptibility to commonly used oral antimicrobial agents remained at a constant level when *H. influenzae* isolates collected from children in a day-care center during 1979 through 1983 were compared with strains collected during 1984 through 1987.

Haemophilus influenzae is an important bacterial pathogen of the upper respiratory tract during childhood (2). Nonencapsulated (nontypeable) strains are common causes of conjunctivitis, otitis media, and maxillary sinusitis (9, 16, 36). Although these localized mucosal infections are rarely life-threatening, they are associated with significant morbidity; otitis media alone accounts for up to 40% of all pediatric office visits (17, 34).

The prevalence of otitis media appears to be two to four times higher in children attending group day-care centers than it is in children cared for at home or in smaller day-care environments in homes (22, 27, 32, 35). The clinical diagnosis of middle ear infection in children in group day-care centers typically results in empiric therapy with orally administered antimicrobial agents. The frequent use of these antimicrobial agents could contribute to the emergence of antibiotic-resistant pathogens within pediatric populations (13, 31). We recently reported a high rate of nasopharyngeal carriage of antibiotic-resistant pneumococci by children in a day-care center environment (14). In that report, we documented an increasing rate of pneumococcal resistance to trimethoprim-sulfamethoxazole during the 5 years of its use in that setting. Because colonizing *H. influenzae* strains were exposed to the same antibiotic pressures as the previously studied pneumococci, we were concerned that similar resistance trends could be occurring in *H. influenzae* carried by children in this day-care center population. The present investigation was undertaken to determine, in a longitudinal fashion, the prevalence of resistance to commonly used antimicrobial agents in *H. influenzae* strains carried by these attendees of a day-care center.

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MATERIALS AND METHODS

Day-care study population. The isolates of *H. influenzae* studied were recovered from nasopharyngeal secretions of children attending the Frank Porter Graham Child Development Center. Details of the recruitment of these children and of the longitudinal design of respiratory infection surveillance conducted in this population have been published in previous reports (11, 12). Briefly, a cohort of 10 to 12 infants 2 to 3 months of age was admitted to the day-care program each year. Children attended the center for 8 h a day, 5 days a week, 50 weeks a year. Approximately 80% of the children enrolled were followed continuously in this setting from early infancy to entrance into kindergarten.

Recovery of isolates. Bacterial surveillance cultures of upper respiratory tract secretions were performed each month. In addition, samples of upper respiratory tract secretions were cultured for viruses and bacteria at the onset of each respiratory illness. Respiratory secretions were obtained by performing a saline nasal wash. Aliquots of the wash fluid were streaked onto sheep blood and chocolate agar which in turn were incubated at 37°C in 5% CO₂. Isolates of *H. influenzae* were identified by characteristic colony morphology, Gram stain morphology, and hemin and NAD requirement by using X-, V-, and XV-factor paper strips (BBL, Cockeysville, Md.) (19). In addition, all of the isolates were examined for capsular antigens by a slide agglutination procedure by using the Bacto *H. influenzae* antisera set (Difco Laboratories, Detroit, Mich.).

The test strains used in this study were isolated during the

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years 1979 through 1987 from 86 study children between 2 months and 3 years of age. Test strains collected during the 9-year study period were stored in skim milk at -70°C . Antimicrobial susceptibility tests were performed in 1988 and 1989 by using these stored isolates.

Selection of isolates for susceptibility testing. Chronological records of bacterial culture results for each child enrolled in the center were used for the test isolate selection process. Test strains were chosen such that there was a time period of at least 90 days between *H. influenzae* isolations when multiple strains from the same child were studied.

Susceptibility testing of isolates. All isolates were tested for β -lactamase production by using chromogenic cephalosporin (nitrocefin) reagent (Glaxo, Greenford, England) (28). In addition, MIC antimicrobial susceptibility testing was performed by using agar dilution methodology (37). For amoxicillin (Beecham Laboratories, Bristol, Tenn.), cefaclor (Eli Lilly & Co., Indianapolis, Ind.), cefuroxime sodium (Glaxo Laboratories Ltd., Greenford, England), and chloramphenicol (Parke-Davis, Morris Plains, N.J.), serial twofold dilutions ranging from 0.03 to 16 $\mu\text{g/ml}$ were incorporated into *Haemophilus* Test Medium (HTM) prepared as described by Jorgensen et al. (18). Briefly, HTM consists of Mueller-Hinton agar (Difco Laboratories) with bovine hematin (15 $\mu\text{g/ml}$; Sigma, St. Louis, Mo.), β -NAD (15 $\mu\text{g/ml}$; Kodak, Rochester, N.Y.), and yeast extract (5 mg/ml; Difco Laboratories). Amoxicillin and clavulanic acid were tested at a 2:1 ratio of amoxicillin to potassium clavulanate (Beecham Laboratories). Serial twofold dilutions of trimethoprim (range, 0.03 to 8 $\mu\text{g/ml}$; Burroughs-Wellcome Co., Research Triangle Park, N.C.) and sulfamethoxazole (range, 0.59 to 152 $\mu\text{g/ml}$; Burroughs-Wellcome Co.) were incorporated alone and in 1:19 combinations into HTM supplemented with thymidine phosphorylase (0.2 IU/ml; Burroughs-Wellcome Co.). Erythromycin (range, 0.03 to 32 $\mu\text{g/ml}$; Abbott Laboratories, North Chicago, Ill.) and sulfisoxazole (range, 2.0 to 512 $\mu\text{g/ml}$; Sigma) also were added alone and in 1:64 combinations into HTM containing thymidine phosphorylase. A 1:64 ratio of these two agents was chosen for study on the basis of the results of in vitro studies by Whitley and Pelton (38) and in vivo drug level studies by Howard et al. (15).

Test inocula were prepared by suspending colonies from an overnight growth of each isolate into Trypticase soy broth to equal the turbidity of a no. 0.5 McFarland standard. The standardized suspensions then were diluted 1:10 in Trypticase soy broth. Plates were inoculated with suspensions of test strains (approximately 10^4 CFU per spot) by using a Steers replicator (30). The inoculated HTM plates were incubated at 35°C in CO_2 for 18 to 24 h. The MIC was defined as the lowest concentration of antimicrobial agent at which growth was completely inhibited; a very fine, barely visible haze or a single colony was not regarded as growth. When trimethoprim and sulfonamides were tested, the endpoint was taken as the lowest concentration of antimicrobial agent demonstrating a sudden, sharp (80 to 90%) decrease in growth (19). *Escherichia coli* ATCC 25922 and *Streptococcus faecalis* ATCC 29212 were used as controls for each experiment. The expected MICs for these two organisms were observed for all the antimicrobial agents tested. MICs were determined in duplicate on separate plates and on separate days. The higher MIC of the duplicate values was used for data analysis. None of the duplicate results differed by more than one twofold dilution.

TABLE 1. Prevalence of β -lactamase production in 217 *H. influenzae* isolates recovered during 1979 through 1987

Classification	1979-1983		1984-1987	
	No. of strains tested	No. (%) of strains β -lactamase positive	No. of strains tested	No. (%) of strains β -lactamase positive
Total	104	19 (18)	113	20 (18)
Type b	15	11 (73)	6	1 (17)
Type e	0	0 (0)	10	1 (10)
Type f	2	0 (0)	12	0 (0)
Nontypeable	87	8 (9)	85	18 (21)

RESULTS

A total of 217 isolates of *H. influenzae* recovered between 1979 and 1987 from a total of 86 children attending the Frank Porter Graham Child Development Center were examined for their in vitro susceptibilities to a panel of 11 antimicrobial agents. The average number of isolates tested was 2.5 per child (range, 1 to 7). Among the 217 isolates, 10% were serologically classified as type b, 5% as type e, 6% as type f, and 79% as nontypeable.

The ability of *H. influenzae* isolates to produce β -lactamase was determined by using the chromogenic cephalosporin nitrocefin. Table 1 summarizes the prevalence of β -lactamase production and the distribution of that production by isolate serotype. One hundred four of the strains tested were recovered during 1979 through 1983. Nineteen (18%) of these strains were positive for β -lactamase production. Seventy-three percent of the 15 type b strains recovered in this time period produced β -lactamase. None of the type e or f strains and 9% of the 87 nontypeable strains were β -lactamase positive. One hundred thirteen strains were recovered during the 1984 through 1987 time period, and 20 or, again, 18% of those isolates were positive for β -lactamase production. Seventeen percent of the 6 type b strains, 10% of the type e strains, 0% of the type f strains, and 21% of the 85 nontypeable strains were positive for β -lactamase production in the later time period.

The in vitro susceptibility of *H. influenzae* to four beta-lactam agents is shown in Table 2. The MICs of amoxicillin for 50% (MIC_{50}) and 90% (MIC_{90}) of the β -lactamase-positive strains tested were >16.0 $\mu\text{g/ml}$. Amoxicillin MICs

TABLE 2. MICs of four beta-lactam agents against 39 β -lactamase-positive strains and 178 β -lactamase-negative strains of *H. influenzae* recovered during 1979 through 1987

Antimicrobial agent	β -Lactamase	MIC ($\mu\text{g/ml}$) ^a		
		50%	90%	Range
Amoxicillin	Positive	>16.0	>16.0	4.0-16.0
	Negative	1.0	1.0	0.125-1.0
Amoxicillin-clavulanate ^b	Positive	1.0	2.0	0.5-4.0
	Negative	0.5	1.0	0.125-1.0
Cefaclor	Positive	8.0	16.0	2.0- >16.0
	Negative	4.0	16.0	1.0- >16.0
Cefuroxime	Positive	0.5	1.0	0.25-2.0
	Negative	0.5	1.0	0.125-2.0

^a 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

^b This combination was tested at a constant ratio of 2 parts of amoxicillin to 1 part of clavulanic acid. The concentration indicated is the concentration of amoxicillin.

TABLE 3. Susceptibility summary of seven antimicrobial agents against 217 strains of *H. influenzae* recovered during 1979 through 1987

Antimicrobial agent	MIC ($\mu\text{g/ml}$) ^a		
	50%	90%	Range
Chloramphenicol	0.5	1.0	0.06–4.0
Trimethoprim	0.5	4.0	0.06–8.0
Sulfamethoxazole	>152.0	>152.0	19.0–>152.0
Trimethoprim-sulfamethoxazole ^b	0.125/2.38	0.25/4.76	$\leq 0.03/0.59$ –1.0/19.0
Erythromycin	4.0	8.0	0.25–16.0
Sulfisoxazole	128.0	128.0	8.0–256.0
Erythromycin-sulfisoxazole ^c	2.0/128.0	2.0/128.0	0.25/16.0–4.0/256.0

^a 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

^b This combination was tested at a constant ratio of 1 part of trimethoprim to 19 parts of sulfamethoxazole.

^c This combination was tested at a constant ratio of 1 part of erythromycin to 64 parts of sulfisoxazole.

for all strains lacking β -lactamase activity were $\leq 1.0 \mu\text{g/ml}$. All strains for which amoxicillin MICs were $> 1.0 \mu\text{g/ml}$ were β -lactamase positive.

Clavulanic acid was effective in vitro in inhibiting β -lactamase activity, as indicated by the reduction of amoxicillin MICs when a 2:1 combination of amoxicillin and clavulanic acid was used to test β -lactamase-positive strains.

Cefuroxime was found to be more active against the *H. influenzae* isolates tested than was cefaclor, with MIC₉₀s of 1.0 and 16.0 $\mu\text{g/ml}$, respectively. The cefaclor MIC was $\leq 1.0 \mu\text{g/ml}$ for only one test isolate, while for more than 90% of the test isolates, cefuroxime MICs were $\leq 1.0 \mu\text{g/ml}$. As indicated in Table 2, *H. influenzae* susceptibility to cephalosporins was not significantly influenced by β -lactamase production.

The MICs of other antimicrobial agents for *H. influenzae* are presented in Table 3. Chloramphenicol MICs were 4.0 $\mu\text{g/ml}$ for three isolates (1%), while chloramphenicol MICs ranged from 0.06 to 1.0 $\mu\text{g/ml}$ for the remaining strains. The three strains with reduced susceptibility to chloramphenicol were recovered from two of the children in the study, were serologically nontypeable, and were β -lactamase positive.

Trimethoprim-sulfamethoxazole MICs ranged from $\leq 0.03/0.59$ to 1.00/19.0 $\mu\text{g/ml}$, with a median MIC of 0.125/2.38 $\mu\text{g/ml}$. Trimethoprim-sulfamethoxazole MICs for only 2 (<1%) of the 217 isolates studied were $> 0.5/9.5 \mu\text{g/ml}$. The

two strains for which the MICs were increased were recovered from different children, were serotype b, were β -lactamase positive, and were susceptible to chloramphenicol. Trimethoprim MICs were $\leq 0.5 \mu\text{g/ml}$ for 65% of the *H. influenzae* strains when they were tested against this antimicrobial agent alone. None of the isolates was inhibited by sulfamethoxazole alone at concentrations of $\leq 9.5 \mu\text{g/ml}$. Antimicrobial synergy was displayed against 54% of the *H. influenzae* strains tested; MICs of the two drugs in combination were greater than or equal to fourfold lower than the MICs of trimethoprim and sulfamethoxazole when they were tested separately against these isolates.

Erythromycin-sulfisoxazole MICs were $\leq 0.5/32 \mu\text{g/ml}$ for only 5% of the isolates studied. High concentrations of erythromycin and sulfisoxazole alone were required to inhibit the majority of strains tested. Erythromycin MICs were $\leq 0.5 \mu\text{g/ml}$ for only 2% of the isolates studied, while sulfisoxazole MICs were $\leq 32 \mu\text{g/ml}$ for 3% of the isolates studied when they were tested against each of these two drugs alone. Although the combination of erythromycin-sulfisoxazole was more active than erythromycin alone, the combination had activity essentially equal to that of sulfisoxazole alone, with synergy demonstrated in only 0.5% of the isolates tested.

Comparison of MIC₅₀ and MIC₉₀ data for 1979 through 1983 and 1984 through 1987 indicated no significant differences in susceptibilities to the antimicrobial agents during the two time periods studied. In addition, the proportion of isolates susceptible to concentrations of antimicrobial agents usually achieved in middle ear fluid remained consistent over the period of study (Table 4).

DISCUSSION

Recent reports have suggested that day-care center attendance and trends in antibiotic use in children attending day-care centers can influence the epidemiology of antimicrobial resistance in pediatric bacterial pathogens (13, 31). We previously conducted an antibiotic resistance survey for pneumococci in a day-care population and found periods of time when the prevalence of pneumococci with reduced susceptibilities to both penicillin G and trimethoprim-sulfamethoxazole was relatively high (14). We undertook the present investigation to survey the extent of antibiotic resistance in strains of *H. influenzae* colonizing the upper respiratory tracts of children attending the same day-care center. Because otitis media was the most common illness requiring

TABLE 4. MICs and percent susceptibility of *H. influenzae* isolates recovered during 1979 through 1983 and 1984 through 1987 to commonly used oral antimicrobial agents

Antimicrobial agent	Concn achievable in MEF ($\mu\text{g/ml}$) ^a	1979–1983			1984–1987		
		MIC ($\mu\text{g/ml}$) ^b		% Susceptible ^c	MIC ($\mu\text{g/ml}$)		% Susceptible
		50%	90%		50%	90%	
Amoxicillin	1.0	0.5	>16.0	82	0.5	16.0	82
Amoxicillin-clavulanate	1.0/0.5	0.5/0.25	1.0/0.5	88	0.5/0.25	1.0/0.5	97
Cefaclor	1.0	8.0	16.0	0	4.0	8.0	1
Cefuroxime	1.0	0.5	1.0	91	0.5	1.0	94
Trimethoprim-sulfamethoxazole	0.5/9.5	0.125/2.38	0.25/4.76	98	0.125/2.38	0.25/4.76	100
Erythromycin	0.5 ^d	4.0	8.0	2	4.0	8.0	2
Erythromycin-sulfisoxazole ^d	0.5/32	2.0/128	2.0/128	2	2.0/128	2.0/128	7

^a Concentration of antimicrobial agent usually achieved in middle ear fluid (MEF). See text for references.

^b 50% and 90%, MICs for 50 and 90% of isolates tested, respectively.

^c Percent susceptible to less than or equal to the concentration of antimicrobial agent usually achieved in middle ear fluid.

^d Erythromycin concentration in middle ear fluid when administered as ethylsuccinate.

antibiotic treatment in this group of children, susceptibility results from this study were based on the concentrations of antimicrobial agents achievable in middle ear fluid.

The overall rate of β -lactamase production for all the isolates investigated in this study was 18%. β -Lactamase production was documented in 57% of all the type b isolates and 14% of the total non-type b isolates tested. On the basis of a comparison of the β -lactamase positivity of total isolates from the first 5 years of our study with that of the last 4 years, the prevalence of β -lactamase-producing *H. influenzae* strains did not increase in our day-care population. The serotype distribution of β -lactamase production did appear to change; however, the number of organisms tested was too small to determine the significance of this observation.

β -Lactamase activity was inhibited, and reductions in the amoxicillin MICs were demonstrated when β -lactamase-positive isolates were tested for their susceptibility to amoxicillin in combination with the β -lactamase inhibitor clavulanic acid. However, for only 56% of these isolates tested against this combination were amoxicillin MICs reduced to the levels seen for β -lactamase negative strains or to levels readily achieved in middle ear fluid (≤ 1.0 $\mu\text{g/ml}$) (10, 15, 21, 26).

There was no apparent trend of increasing resistance to cefaclor or cefuroxime among the isolates examined in the present investigation. Of interest, however, was that cefuroxime MICs were ≤ 1.0 $\mu\text{g/ml}$ for 201 (93%) of the 217 *H. influenzae* strains tested, while cefaclor MICs were ≤ 1.0 $\mu\text{g/ml}$ for less than 1% of the isolates; ≤ 1.0 $\mu\text{g/ml}$ is the concentration of these cephalosporin agents usually attained in middle ear fluid (7, 21, 23, 24, 26). Our findings regarding cefaclor are compatible with those of other investigators. In a 1978 study of clinical *H. influenzae* isolates, fewer than 10% of the strains were inhibited by a concentration of 0.78 μg of cefaclor per ml (29). In a more recent national survey of 2,811 clinical isolates, only 5% were inhibited by cefaclor at a concentration of ≤ 1.0 $\mu\text{g/ml}$ (6). In contrast to cefaclor, the cefuroxime MIC₉₀ for 100 clinical isolates in a 1983 study was 1.0 $\mu\text{g/ml}$ (8).

H. influenzae strains for which chloramphenicol MICs were > 2.0 $\mu\text{g/ml}$ were uncommon (1%) in the strains from the day-care population that we studied, as they have been in recent national surveys (5, 6). Since 1972, chloramphenicol resistance has been reported in both type b and non-type b *H. influenzae* clinical isolates. A large proportion of these clinical strains also have been resistant to ampicillin, tetracycline, and trimethoprim-sulfamethoxazole (3, 25). All three of our strains for which chloramphenicol MICs were increased were non-type b and β -lactamase positive, but they were trimethoprim-sulfamethoxazole susceptible. Tetracycline was not tested against our study isolates.

The antimicrobial combination trimethoprim-sulfamethoxazole was highly active in vitro against our *H. influenzae* study strains at concentrations achievable in middle ear fluid, $\leq 0.5/9.5$ $\mu\text{g/ml}$ (20, 21). Antibiotic synergy was demonstrated against 54% of the study isolates tested against this combination. In studies by other investigators, $\geq 99\%$ of both clinical isolates and survey isolates from healthy children were susceptible to $\leq 0.5/9.5$ μg of trimethoprim-sulfamethoxazole per ml (4, 6, 29). The opposite was true in our previous experience with pneumococci (14); the use of trimethoprim-sulfamethoxazole resulted in the rapid selection of strains with reduced susceptibilities to this drug combination. Before the introduction of trimethoprim-sulfamethoxazole in 1981, pneumococcal resistance to this drug

was only 5% in the day-care study population. Resistance rose to 39%, however, during the 5 years following the introduction of this antimicrobial agent. Of interest is the fact that the *H. influenzae* isolates we tested in the present study had experienced the same antibiotic pressures as the previously tested pneumococcal isolates, yet there was no increase in the prevalence of resistance to trimethoprim-sulfamethoxazole in the *H. influenzae* isolates examined in this study.

High concentrations of both erythromycin and sulfisoxazole alone and in combination were required to inhibit the majority of *H. influenzae* strains studied in the present investigation. On the basis of the current literature, probably no more than 0.5 μg of erythromycin per ml is achievable in the middle ear when it is given in the form of erythromycin ethylsuccinate (1, 10, 26, 33); 98% of the isolates we tested were not susceptible to this concentration of erythromycin in vitro. In a recent national survey, comparable results were observed, in which erythromycin MICs were ≤ 0.5 $\mu\text{g/ml}$ for only 1% of the strains tested (6).

Erythromycin ethylsuccinate-sulfisoxazole is a commonly prescribed combination therapy for otitis media (1, 10, 26, 33). However, in this investigation we found a very high prevalence (95%) of *H. influenzae* strains that were not susceptible to this combination in vitro when $\leq 0.5/32.0$ μg of erythromycin-sulfisoxazole per ml was used as the criterion for susceptibility. In another study that tested the same ratio of these two drugs, only 37% of the *H. influenzae* isolates were inhibited by $\leq 0.5/32$ μg of the combination per ml (32). Also, in our investigation we found little evidence that the combination of erythromycin and sulfisoxazole (1:64) is synergistic against those isolates tested.

In this investigation, we studied in a longitudinal fashion the in vitro antimicrobial susceptibility of *H. influenzae* isolates recovered from nasopharyngeal secretions of children known to have high rates of exposure to antibiotics secondary to increased rates of otitis media. The survey was undertaken to determine the prevalence of resistance to antimicrobial agents commonly used for the treatment of middle ear infections. In particular, we were interested in trends in resistance to trimethoprim-sulfamethoxazole because increases in resistance to this antimicrobial agent were previously documented in pneumococci carried by children in this same population. In summary, many of the *H. influenzae* isolates tested were not susceptible to the antimicrobial concentrations achievable in middle ear fluid; however, there was no observable trend toward resistance to trimethoprim-sulfamethoxazole or to any of the other oral antimicrobial agents studied in this investigation.

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