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Antimicrobial Susceptibility of *Haemophilus influenzae* in the Respiratory Tracts of Patients with Cystic Fibrosis

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We analyzed the antimicrobial susceptibilities of *Haemophilus influenzae* isolates from 157 sputum specimens prospectively collected from 39 cystic fibrosis (CF) patients during a 2-year study. These isolates were characterized by random amplified polymorphic DNA analysis and major outer membrane protein (MOMP) analysis to identify *H. influenzae* strains and MOMP variants and to assess their persistence in the respiratory tract. Among the 247 *H. influenzae* isolates, 16 (6.5%) produced β-lactamase. The 231 β-lactamase-negative isolates represented 85 *H. influenzae* strains, 61 MOMP variants derived from 27 of these strains, and 85 persistent isolates identical to strains or MOMP variants. All β-lactamase-negative isolates were tested for susceptibility to ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefotaxime, cefaclor, imipenem, tetracycline, and trimethoprim-sulfamethoxazole by disk diffusion testing. Eleven (13%) *H. influenzae* strains, 18 (30%) MOMP variants, and 30 (35%) persistent isolates were resistant to one or more of the antibiotics tested. Antimicrobial susceptibility was decreased among MOMP variants and persistent isolates compared to nonpersistent *H. influenzae* strains, and changes in susceptibility occurred irrespective of MOMP variation. We conclude that the decreased antimicrobial susceptibility of *H. influenzae* during persistence contributes to the poor eradication of *H. influenzae* from the respiratory tracts of CF patients.

Nonencapsulated *Haemophilus influenzae*, although a commensal of the upper respiratory tracts of healthy persons, is an important cause of acute, recurrent, and persistent infections of the human respiratory tract (28). These infections include lower respiratory tract infections of patients with cystic fibrosis (CF) (10, 17, 19).

Data on the antimicrobial susceptibility of *H. influenzae* isolates obtained from CF patients are limited. McCarthy et al. (15) have reported β-lactamase-mediated antimicrobial resistance to ampicillin of *H. influenzae* isolates collected from sputa of CF patients during a 4-year study. However, the persistence of *H. influenzae* strains was not assessed in this study. Later on, by using random amplified polymorphic DNA (RAPD) analysis as a method for genotypic characterization (29) and major outer membrane protein (MOMP) analysis for phenotypic characterization of *H. influenzae* (19), it was found that *H. influenzae* strains and their MOMP variants apparently coexisted in the respiratory tracts of CF patients. Recently, it was reported that these *H. influenzae* strains persisted for periods of up to at least 2 years despite extensive antimicrobial treatment with various antibiotics including β-lactam antibiotics. During the persistence of *H. influenzae* strains, multiple MOMP variants were frequently isolated from CF patients (19).

In this study, we determined the antimicrobial susceptibilities of 247 *H. influenzae* isolates, obtained from 39 CF patients during a 2-year follow-up study, to ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefotaxime, cefaclor, imipenem, tetracycline, and trimethoprim-sulfamethoxazole. We assessed whether MOMP variation within an *H. influenzae* strain affects antimicrobial susceptibility and whether the susceptibilities of persistent strains change over time. In addition, we compared the susceptibilities of persistent isolates and nonpersistent strains without MOMP variation.

MATERIALS AND METHODS

Patients. Forty CF patients (17 females and 23 males) with a history of *H. influenzae* infections who were attending the Academic Medical Center or the Free University Hospital in Amsterdam from 1 May 1990 to 1 January 1991 were included in a 2-year follow-up study. Patients visited either the outpatient department or were admitted to the hospital. The study population consisted of 10 children, 17 adolescents, and 13 adults. The diagnosis of CF was based on a sweat chloride concentration of >70 mmol/liter. The ages of the patients ranged from 0.2 to 36 years (median age, 13 years). The occurrence of exacerbations was recorded throughout the study. Exacerbations were defined as increased sputum production coinciding with a change in the volume and appearance of the sputum in combination with clinical symptoms such as increased cough, fever, weight loss, and decreased exercise tolerance. Patients with an exacerbation received either antibiotic treatment at home or in the hospital, usually for 2 to 3 weeks. The duration of treatment following an exacerbation is determined by the improvement of spirometric indices to previous levels or to a plateau level. Empiric treatment consisted usually of 1- to 2-week courses of various intravenously administered antibiotics followed by orally given antibiotics for 1 to 2 weeks. The choice of the antibiotics was determined by the sensitivity pattern of bacterial isolates obtained from sputum cultures before or during an exacerbation.

Examination of sputum specimens and isolation of *H. influenzae*. A total of 477 sputum specimens were collected in sterile vials at routine outpatient visits or on admission to the hospital. All sputa were expectorated under the supervision of a physiotherapist. In addition, 77 "gagged" sputa were obtained from nine nonexpectorating children after forced coughing induced by a throat swab (19). The median number of sputum specimens per patient was 10.5 (range, 3 to 35). Sputa were stored at 4°C within 30 min of collection. A sputum specimen was washed twice in phosphate-buffered saline (pH 7.2) and was microscopically examined within 4 h. The Gram stain was used to exclude sputa contaminated with oropharyngeal flora. *H. influenzae* was isolated by using selective media including *N*-acetyl-D-glucosamine medium (18) and was identified by its growth requirement for hemin (X factor) and NAD (V factor) (3). Capsular polysaccharide typing was performed with the coagglutination test (5).

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MOMP and RAPD analyses of *H. influenzae* isolates and strain definitions. Based on previous results (19), a value of 20 individual *H. influenzae* colonies per sputum specimen appeared to be representative for detecting the presence of multiple *H. influenzae* strains and/or MOMP variants in the lower respiratory tracts of CF patients. Thus, 20 individual *H. influenzae* isolates obtained as single colonies from primary culture media were phenotypically characterized by MOMP analysis as described previously (9, 19). Whole-cell preparations of *H. influenzae* isolates showing differences in the locations of MOMP P2 (molecular weight, 39,000 to 42,000) and/or P5 (molecular weight, 37,000 to 39,000) on a sodium dodecyl sulfate-polyacrylamide gel 10 cm in length after protein staining were regarded as distinct. Isolates with distinct MOMP patterns were further genotypically characterized by RAPD analysis to discriminate *H. influenzae* strains from MOMP variants (29). In short, genomic DNA was amplified with random primers ERIC1R and ERIC2. PCR was performed at an extension temperature of 74°C (4 min) for 35 cycles. One *H. influenzae* strain was used as a reference strain for each PCR run.

H. influenzae strains are strains with unique MOMP and RAPD compositions since they differ in both their MOMP and RAPD patterns, whereas MOMP variants differ only in their MOMP patterns, not in their RAPD patterns (19, 29). *H. influenzae* strains with identical MOMP patterns obtained from various sputum specimens always showed identical RAPD patterns (19). We assumed that the first isolate of *H. influenzae* isolates with an identical RAPD pattern but showing variation in MOMP pattern was the original strain from which MOMP variants were derived (19). An *H. influenzae* strain or MOMP variant reisolated from respiratory tract specimens at least 1 month after isolation of a previous strain or MOMP variant with identical RAPD and MOMP patterns was regarded as being persistent.

Determination of β-lactamase activity. *H. influenzae* isolates were tested for β-lactamase activity by the chromogenic cephalosporin test with nitrocephin as the substrate (22).

Antimicrobial susceptibility testing. The disk diffusion susceptibility test for *H. influenzae* was performed on standard haemophilus test medium (diameter, 9 cm; Becton Dickinson, Erembodegem, Belgium) as described by Doern et al. (7). *H. influenzae* isolates were cultured on chocolate agar medium, supplemented with 10 μg of hemin (X factor) and 10 μg of NAD (V factor) per ml at 37°C in air enriched with 5% CO_2 for 18 h. Slowly growing isolates were incubated for another 18 h. A bacterial suspension was prepared in phosphate-buffered saline (pH 7.2) corresponding in density to McFarland standard 0.5. This suspension yielded 1.6×10^8 to 8×10^8 CFU/ml, as determined by colony counting. Antimicrobial susceptibility was tested with Sensi-discs (Becton Dickinson), according to the method described by National Committee for Clinical Laboratory Standards (NCCLS) standard M2-A4 (20). The antibiotics included ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefotaxime, cefaclor, imipenem, tetracycline, and trimethoprim-sulfamethoxazole. Zones of inhibition were measured to the nearest millimeter on single trials. Results were interpreted by using the criteria contained in NCCLS standard M2-A4 (20).

Statistics. Statistical analysis of antimicrobial susceptibility for various groups of non-β-lactamase-producing *H. influenzae* strains was performed by a chi-square analysis. P values of <0.05 were considered significant.

RESULTS

In total, 247 nonencapsulated *H. influenzae* isolates were obtained from 157 of 554 (28.3%) sputum specimens from 39 CF patients. Similar results have been described previously (19). The sputum cultures of 1 CF patient remained *H. influenzae* negative. The *H. influenzae* isolates represented 94 *H. influenzae* strains and 66 MOMP variants derived from 30 of these strains. A total of 87 isolates identical to 11 *H. influenzae* strains and 38 MOMP variants were persistent. *H. influenzae* strains from all CF patients were distinct, except for those from two pairs of siblings; the two children of each pair had MOMP variants of the same *H. influenzae* strain, but the strains of each pair were different.

Sixteen of the 247 (6.5%) *H. influenzae* isolates obtained from eight patients produced β-lactamase. These β-lactamase-producing isolates consisted of nine *H. influenzae* strains, five MOMP variants, and two persistent isolates identical to two MOMP variants. All β-lactamase-producing *H. influenzae* isolates were susceptible to amoxicillin-clavulanic acid, cefuroxime, cefotaxime, and imipenem. Eight isolates were intermediate or resistant to one or more of the antibiotics cefaclor, tetracycline, and trimethoprim-sulfamethoxazole. All β-lactamase-producing *H. influenzae* isolates were resistant to ampicillin. The number of β-lactamase-producing *H. influenzae* isolates was too low for further comparisons.

TABLE 1. Antimicrobial susceptibilities of all 231 non-β-lactamase-producing *H. influenzae* isolates compared to 85 *H. influenzae* strains obtained from 39 CF patients during a 2-year follow-up study

Antibiotic(s)	No. (%) of non-β-lactamase-producing *H. influenzae*[a]:					
	Isolates (n = 231) that were:			Strains (n = 85) that were:		
	S	I	R	S	I	R
Ampicillin	212 (92)	11 (5)	8 (3)	82 (97)	2 (2)	1 (1)
Amoxicillin-clavulanic acid	216 (94)	0 (0)	15 (6)	84 (99)	0 (0)	1 (1)
Cefaclor	192 (83)	19 (8)	20 (9)	83 (98)	1 (1)	1 (1)
Cefuroxime	217 (94)	6 (3)	8 (3)	83 (98)	1 (1)	1 (1)
Cefotaxime	231 (100)	0 (0)	0 (0)	85 (100)	0 (0)	0 (0)
Imipenem	231 (100)	0 (0)	0 (0)	85 (100)	0 (0)	0 (0)
Tetracycline	231 (100)	0 (0)	0 (0)	85 (100)	0 (0)	0 (0)
Trimethoprim-sulfamethoxazole	214 (93)	0 (0)	17 (7)	78 (92)	0 (0)	7 (8)

[a] S, susceptible to the antibiotic tested; I, intermediate to the antibiotic tested; R, resistant to the antibiotic tested.

Susceptibility testing was performed on all 231 non-β-lactamase-producing *H. influenzae* isolates. These isolates represented 85 *H. influenzae* strains and 61 MOMP variants derived from 27 of these strains. A total of 85 isolates identical to 11 *H. influenzae* strains and 36 MOMP variants were persistent. The susceptibilities to ampicillin, amoxicillin-clavulanic acid, cefaclor, cefuroxime, cefotaxime, imipenem, tetracycline, and trimethoprim-sulfamethoxazole are shown in Table 1. In total, 47 of 231 (20%) *H. influenzae* isolates were intermediate or resistant to one or more of the β-lactam antibiotics ampicillin, amoxicillin-clavulanic acid, cefuroxime, and cefaclor.

The 231 non-β-lactamase-producing *H. influenzae* isolates were further analyzed to compare antimicrobial susceptibilities among *H. influenzae* strains (different RAPD and MOMP patterns), MOMP variants (identical RAPD and different MOMP patterns), and persistent isolates (multiple isolates of strains and/or MOMP variants). The susceptibilities of the 85 *H. influenzae* strains were separately analyzed to exclude the effect of MOMP variants and persistent isolates. The susceptibilities of *H. influenzae* strains to the antibiotics tested are shown in Table 1. Four (5%) strains were intermediate or resistant to one or more of the β-lactam antibiotics ampicillin, amoxicillin-clavulanic acid, cefaclor, and cefuroxime.

The results presented in Table 1 revealed that the percentage of strains or isolates with decreased susceptibility (intermediate or resistant) to one or more of the antibiotics ampicillin, amoxicillin-clavulanic acid, cefaclor, and cefuroxime was 4 times higher for the 231 *H. influenzae* isolates (20%) than for the 85 *H. influenzae* strains (5%). In contrast, the percentages of isolates and strains with decreased susceptibility to trimethoprim-sulfamethoxazole were similar (17 of 231 (7%) versus 7 of 85 (8%), respectively). These results indicate that the decreased susceptibility to β-lactam antibiotics among the 231 *H. influenzae* isolates is due to the presence of less-susceptible (intermediate or resistant) MOMP variants derived from susceptible strains and/or the overrepresentation of less-susceptible persistent *H. influenzae* isolates.

In order to analyze whether MOMP variation within a strain affects susceptibility, the susceptibilities of 61 MOMP variants were compared with the susceptibilities of their corresponding 27 *H. influenzae* strains obtained from 20 patients. These *H. influenzae* strains and their MOMP variants were all susceptible to the antibiotics cefotaxime, imipenem, and tetracycline. Eighteen (67%) *H. influenzae* strains and 43 (70%) MOMP

TABLE 2. Antimicrobial susceptibilities of 18 MOMP variants derived from 8 original *H. influenzae* strains from seven patients showing decreased susceptibility to one or more of the antibiotics ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefaclor, and trimethoprim-sulfamethoxazole during a 2-year follow-up study

Patient	MOMP variants[a]	Time points (mo)	Results[b] for:									
			Ampicillin		Amox.-clav. acid		Cefuroxime		Cefaclor		Trim.-sulfa	
			Suscept.	Inh. zone (mm)	Suscept.	Inh. zone (mm)	Suscept.	Inh. zone (mm)	Suscept.	Inh. zone (mm)	Suscept.	Inh. zone (mm)
1	5a	21	S	34	S	26	S	29	S	34	S	40
	5b	21	S	27	S	27	S	26	S	32	R	0
5	15a	1	S	33	S	33	S	35	S	30	S	31
	15b	1	S	33	S	35	S	34	S	30	R	0
10	30a	2	S	31	S	28	S	27	S	27	S	34
	30b	2	S	26	S	28	S	25	I	19	S	34
24	61a	6	S	31	S	29	S	28	S	21	R	0
	61b	6	S	31	S	30	S	30	I	18	R	0
	61c	6	S	29	S	28	S	27	S	22	R	0
27	67a	1	I	23	S	26	S	34	S	21	S	35
	67b	1	S	24	S	21	S	30	R	0	S	42
	67d	7	R	16	R	16	I	22	R	0	S	29
	68a	7	S	32	S	30	S	30	S	29	S	36
	68c	7	S	32	S	32	S	34	S	30	R	0
28	70a	1	R	14	R	19	S	20	S	22	S	24
	70b	1	R	18	R	18	S	24	I	18	S	20
	70c	1	R	15	R	17	S	42	R	0	R	0
	70e	9	R	16	R	17	S	35	S	21	S	26
30	77a	1	S	23	S	23	I	23	I	19	S	33
	77c	1	I	20	R	19	S	25	I	18	S	33
	77d	1	S	23	S	23	S	25	R	0	S	33
	77e	1	S	23	R	13	I	21	R	0	S	32
	77f	1	S	28	S	24	S	28	I	18	S	33
	77g	1	S	24	S	25	S	29	I	19	S	30
	77i	3	S	24	R	18	S	28	S	20	S	32
	77k	14	S	23	S	23	S	28	I	19	S	32

[a] Numbers indicate distinct *H. influenzae* strains, and added letters indicate MOMP variants.
[b] Suscept., susceptibility of persistent *H. influenzae* isolates to the tested antibiotics. S, susceptible to the antibiotic tested; I, intermediate to the antibiotic tested; R, resistant to the antibiotic tested. Inh. zone, inhibition zone for the tested antibiotic. Amox.-clav. acid, amoxicillin-clavulanic acid; trim.-sulfa, trimethoprim-sulfamethoxazole.

variants were susceptible to the other antibiotics tested. Decreased susceptibility was found for 18 (30%) MOMP variants derived from 8 *H. influenzae* strains from seven patients (Table 2). A similar frequency (30%) of decreased susceptibility was found among the 27 original *H. influenzae* strains and their MOMP variants. In general, most of the MOMP variants showed no changes in susceptibility compared to their original *H. influenzae* strains (Table 2). However, changes in the susceptibilities of MOMP variants resulted more frequently in decreased susceptibility than in increased susceptibility, compared to the susceptibilities of their original *H. influenzae* strains. Furthermore, we determined whether decreased susceptibility was limited to MOMP variants or also occurred in nonpersistent *H. influenzae* strains without MOMP variation. Five of 47 (11%) nonpersistent *H. influenzae* strains without MOMP variation showed decreased susceptibility to one or more of the antibiotics tested (these strains were only resistant to trimethoprim-sulfamethoxazole and were not resistant to β-lactam antibiotics). Decreased susceptibility occurred more frequently in *H. influenzae* strains with MOMP variation (8 of 27) than in nonpersistent *H. influenzae* strains without MOMP variation (5 of 47) ($\chi^2 = 4.3$; $P = 0.04$). Therefore, changes in antimicrobial susceptibility occurred irrespective of MOMP variation.

To determine whether changes in susceptibility are associated with persistent *H. influenzae*, we examined whether intermediate and resistant *H. influenzae* strains and their MOMP variants were overrepresented among the 231 *H. influenzae* isolates due to relatively more frequent isolation of these isolates over time. Therefore, the susceptibility of the first isolate of a persistent *H. influenzae* strain or MOMP variant was compared with those of subsequent persistent isolates. Eleven *H. influenzae* strains were reisolated 27 times and 36 MOMP variants were reisolated 58 times from 18 patients. All 85 persistent isolates were susceptible to the antibiotics cefotaxime, imipenem, and tetracycline. The susceptibilities of 10 of 47 (21%) first isolates were decreased for one or more of the β-lactam antibiotics, and one isolate was resistant to trimethoprim-sulfamethoxazole. Of the subsequent persistent isolates, 29 of 85 (34%) showed decreased susceptibility to one or more of the β-lactam antibiotics and 3 (4%) were resistant to trimethoprim-sulfamethoxazole.

Variation in susceptibility was observed for 12 persistent isolates of one *H. influenzae* strain and 28 persistent isolates of 11 MOMP variants obtained from five patients. The susceptibilities and zones of inhibition of these 52 persistent isolates for ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefaclor, and trimethoprim-sulfamethoxazole are shown in Table 3. The frequency of decreased susceptibility to one or more of the antibiotics tested among persistent isolates (30 of 85) did not differ from that among the first isolates (11 of 47) ($\chi^2 = 2.0$; $P = 0.16$). This indicates that the first isolated *H. influenzae* strains or MOMP variants and their subsequent isolates during persistence have similarly decreased susceptibilities. During antibiotic treatment of the patients, the susceptibilities of some sequentially isolated persistent *H. influenzae* isolates increased and some decreased over time. The cumulative duration of antibiotic treatment of the 18 patients with persistent *H. influenzae* isolates (623 weeks; mean, 35 ± 25 weeks) did not differ from that of 25 patients with nonpersistent *H. influenzae* strains without MOMP variation (656 weeks; mean 26 ± 19 weeks) (Mann-Whitney test; $P = 0.14$).

These results suggested that persistent *H. influenzae* isolates were more resistant than nonpersistent *H. influenzae* strains without MOMP variation. Therefore, the susceptibilities of persistent isolates and nonpersistent strains were compared. Thirty of 85 (35%) persistent isolates showed decreased susceptibility to one or more of the antibiotics tested. In contrast, 5 of 47 (11%) nonpersistent strains showed decreased susceptibility to these antibiotics (these strains were only resistant to trimethoprim-sulfamethoxazole and not resistant to β-lactam antibiotics). The frequency of decreased susceptibility was higher for persistent isolates than for nonpersistent *H. influenzae* strains ($\chi^2 = 9.4$; $P = 0.002$).

DISCUSSION

In the present study, 6.5% of the nonencapsulated *H. influenzae* isolates obtained from sputum specimens of 39 CF patients during a 2-year study were β-lactamase positive. A higher frequency of β-lactamase-positive strains, ranging from 8.3 to 15.6%, has been reported for *H. influenzae* isolates obtained from patients with various diseases (1, 6, 11, 13, 14, 25, 26). In a study by McCarthy et al. (15), it was demonstrated that 13% of isolates from CF patients produced β-lactamase and were therefore ampicillin resistant, whereas ampicillin resistance of *H. influenzae* strains without β-lactamase activity was not observed. This is in contrast to the results of our study where 8.2% of β-lactamase-negative *H. influenzae* isolates had decreased susceptibility to ampicillin. Higher (12.1%) as well as lower (1%) percentages of β-lactamase-negative *H. influenzae* isolates which were resistant to ampicillin have been reported for clinical isolates from patients with a variety of respiratory diseases (1, 6, 11, 13, 14, 25, 26). The rates of resistance to cefaclor (16.2%) and cefuroxime (5.7%) were similar to those reported by James et al. (13) for *H. influenzae* isolates obtained from a variety of respiratory tract specimens. It was reported that in more than 50% of cases the non-β-lactamase-mediated decreased susceptibility of *H. influenzae* to ampicillin was combined with resistance to cefuroxime and/or cefaclor but not to imipenem. Combined resistance to several β-lactam antibiotics has been previously reported for isolates from patients with chronic respiratory disease (12, 24). The frequency of decreased susceptibility of *H. influenzae* isolates to trimethoprim-sulfamethoxazole in our study (7.3%) was similar to the frequency reported by the European Study Group (14). The low percentage of resistance to tetracycline of *H. influenzae* strains was similar to the percentages reported in the British study (26) and in the European study (14). Decreased susceptibility to tetracycline was only found in β-lactamase-positive strains, suggesting conjugative plasmids encoding resistance to tetracycline and ampicillin. In the American study (6), resistance to tetracycline was mostly observed in combination with plasmid-mediated resistance to ampicillin and chloramphenicol.

Our collection of *H. influenzae* isolates was characterized by MOMP and RAPD analysis as described previously (19, 29). *H. influenzae* strains were shown to differ both in MOMP and RAPD patterns. MOMP variants only differing in MOMP patterns were frequently isolated from the lower respiratory tracts of CF patients. In some patients these variants persisted for many months, often concomitantly (19).

The frequency of decreased susceptibility was lower among *H. influenzae* strains than among all 231 *H. influenzae* isolates. Since one obvious reason for this result was that MOMP variants were more resistant than the strains, the susceptibilities of the strains and their MOMP variants were compared. Our analysis showed that the frequency of decreased susceptibility of MOMP variants was similar to that of strains from which MOMP variants were derived. However, *H. influenzae* strains giving rise to MOMP variants appeared more resistant than strains without MOMP variants. As *H. influenzae* strains with MOMP variation differ in susceptibility from those without MOMP variation, they should be considered as a separate population of strains. Therefore, MOMP variation may play a role in the decreased susceptibility. Variation in MOMPs of *H. influenzae* strains obtained from lower respiratory tract specimens of CF patients occurred mainly in MOMP P2 (19). Variation in MOMP P2 (2) and porins of other bacteria (21) have been associated with altered antibiotic susceptibility. Porins form pores through the outer membrane (27), which act as diffusion channels for small hydrophilic solutes including β-lactam antibiotics (2, 4, 21). Alternatively, as the occurrence of MOMP variation requires the persistence of *H. influenzae* (8, 9, 19, 30), strains with MOMP variants may have become more resistant even before the first *H. influenzae* isolate was obtained, i.e., before inclusion of the patient in the study. A dominant role of porin MOMP P2 variation in decreased susceptibility is not very likely, since persistent isolates without variation in MOMP patterns showed similar frequencies of decreased susceptibility. Therefore, we favor the hypothesis that decreased susceptibility to antibiotics, especially β-lactam antibiotics, is mainly associated with persistent *H. influenzae* in the lower respiratory tracts of CF patients. Interestingly, decreased susceptibility to β-lactam antibiotics was mainly confined to four *H. influenzae* strains (strains 66, 67, 70, and 77) and their MOMP variants isolated from four patients during persistence, suggesting that the conditions in the respiratory tracts of these patients favor selection of less-susceptible strains. It appeared that the first isolated strains which persisted were already resistant at the time of isolation from sputum specimens. No relationship to antibiotic treatment was found since the duration of antibiotic treatment during the study did not differ among the 18 patients with persistent *H. influenzae* isolates and the 25 patients with nonpersistent *H. influenzae* strains without MOMP variation.

Antimicrobial susceptibility changed during the persistence of *H. influenzae* strains and MOMP variants. Despite antibiotic therapy, both a decrease and an increase in the susceptibilities of persistent isolates over time were observed. Although these strains and variants persisted, they were not always recovered from each consecutive sputum specimen. Antibiotic therapy probably reduces the load of certain susceptible *H. influenzae* strains, while other strains are allowed to persist in the respi-

TABLE 3. Antimicrobial susceptibilities to ampicillin, amoxicillin-clavulanic acid, cefuroxime, cefaclor, and trimethoprim-sulfamethoxazole of 52 persistent *H. influenzae* isolates obtained from five CF patients at multiple time points during the 2-year follow-up study

Patient	Strain[a]	Time points (mo)	Results[b] for:									
			Ampicillin		Amox.-clav. acid		Cefuroxime		Cefaclor		Trim.-sulfa	
			Suscept.	Inh. zone (mm)	Suscept.	Inh. zone (mm)	Suscept.	Inh. zone (mm)	Suscept.	Inh. zone (mm)	Suscept.	Inh. zone (mm)
26	66	1	I	20	S	26	R	0	R	0	S	33
		4	S	24	S	22	S	28	S	24	S	38
		8	S	23	S	23	S	31	S	24	S	40
		11	S	24	S	24	R	15	R	0	S	34
		12	S	29	S	20	R	0	R	0	S	35
		13	I	21	R	19	R	0	R	0	S	34
		16	S	25	S	22	I	23	R	0	S	36
		18	S	23	S	21	R	0	R	0	S	38
		19	I	19	S	20	R	0	I	17	S	29
		20	R	18	S	20	R	0	I	18	S	29
		21	I	20	S	23	I	22	I	18	S	36
		22	S	24	R	19	R	0	R	0	S	36
		24	S	24	S	23	S	28	I	18	S	34
27	67a	1	I	20	S	21	S	29	S	21	S	35
		3	S	26	S	22	S	30	I	18	R	0
	67b	1	S	24	S	21	S	30	R	0	S	42
		3	I	21	R	0	S	24	R	0	S	21
		7	S	28	S	28	S	38	R	0	R	0
	67d	7	R	16	R	16	I	22	R	0	S	29
		10	S	22	S	24	I	22	R	0	S	39
28	70b	1	I	18	R	18	S	24	I	18	S	20
		9	R	16	R	17	S	26	S	21	S	26
30	77a	1	S	23	S	23	I	23	I	19	S	33
		3	S	23	S	22	S	27	S	20	S	30
		4	I	21	R	0	S	24	R	0	S	28
		6	S	26	R	19	S	25	S	21	S	33
		14	S	30	S	35	S	34	S	23	S	38
	77b	1	S	23	S	23	S	26	S	20	S	41
		3	S	22	S	21	S	25	I	19	S	42
		4	S	23	S	24	S	26	I	18	S	33
	77c	1	I	20	R	19	S	25	I	18	S	33
		3	S	29	S	25	S	29	I	19	S	31
		4	S	23	S	24	S	25	R	0	S	30
		18	S	22	S	21	S	29	S	22	S	32
		20	I	20	S	24	S	29	S	21	S	34
	77e	1	S	23	R	13	I	21	R	0	S	32
		6	I	20	R	19	I	21	R	0	S	34
		16	S	23	S	23	S	28	S	21	S	35
		19	I	21	S	21	S	24	R	0	S	33
		20	S	24	S	26	S	31	S	21	S	38
		21	S	29	S	27	S	35	S	21	S	37
		22	S	28	S	28	S	29	R	0	S	44
		24	S	30	S	30	S	32	S	22	S	44
	77f	1	S	28	S	24	S	28	I	18	S	33
		3	S	24	S	23	S	25	I	18	S	34
		17	S	23	S	24	S	28	S	20	S	32
		18	S	24	S	27	S	30	I	18	S	34
	77i	3	S	24	R	18	S	28	S	20	S	32
		21	S	35	S	31	S	30	S	24	S	33
40	97b	3	S	34	S	35	S	32	S	29	S	34
		4	S	31	S	31	S	32	S	30	R	0
		5	S	32	S	31	S	31	S	30	R	0

[a] Numbers indicate distinct *H. influenzae* strains, and added letters indicate MOMP variants.

[b] Suscept., susceptibility of persistent *H. influenzae* isolates to the tested antibiotics. S, susceptible to the antibiotic tested; I, intermediate to the antibiotic tested; R, resistant to the antibiotic tested. Inh. zone, inhibition zone for the tested antibiotic. Amox.-clav. acid, amoxicillin-clavulanic acid; trim.-sulfa, trimethoprim-sulfamethoxazole.

ratory tract. However, no evidence was found for the gradual increase of resistance among longitudinally obtained *H. influenzae* isolates even after extensive antibiotic treatment (19). It is suggested that selection mechanisms resulting in less-susceptible isolates during persistence are rather ineffective. Other factors such as the low level of penetration of antibiotics into the viscous sputum may also be responsible for the poor eradication of *H. influenzae* from the respiratory tracts of CF patients (23).

Changes in susceptibility to β-lactam antibiotics as well as to other antibiotics were not associated with MOMP P2 variation. Non-β-lactamase-mediated resistance to β-lactam antibiotics may be due to alterations in target proteins for these antibiotics such as penicillin-binding proteins (16). The MOMP P2 variation is therefore most likely antigenic variation, occurring under strong immunological pressure during persistence of *H. influenzae* as previously reported (8, 30).

In conclusion, decreased antimicrobial susceptibility of non-β-lactamase-producing *H. influenzae* isolates occurred more frequently among MOMP variants and persistent strains than among nonpersistent strains. Changes in the susceptibilities of persistent *H. influenzae* strains in the respiratory tracts of CF patients occurred irrespective of MOMP variation.

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