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Nasopharyngeal Carriage of Potential Bacterial Pathogens Related to Day Care Attendance, with Special Reference to the Molecular Epidemiology of *Haemophilus influenzae*

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Nasopharyngeal carriage of *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* was studied in 259 children attending day care centers (DCC) in Amsterdam, The Netherlands, and in 276 control children. The DCC children were sampled a second time after 4 weeks. Carriage rates for DCC children and controls were 58 and 37% for *S. pneumoniae*, 37 and 11% for *H. influenzae*, and 80 and 48% for *M. catarrhalis*, respectively. No increased antibiotic resistance rates were found in strains isolated from DCC children. All *H. influenzae* isolates were typed by random amplified polymorphic DNA (RAPD) analysis. Evidence for frequent transmission of *H. influenzae* strains within DCC was found. In the control group only two isolates (4%) displayed identical RAPD types versus 38% of strains from DCC children. Colonization with *H. influenzae* appeared to be short-lived in these children; more than half of the children harboring *H. influenzae* in the first sample were negative in the second sample, whereas most children still positive in the second sample had a different genotype than in the first sample. Of the newly acquired strains in the second sample, 40% were identical to a strain that had been found in a child in the same DCC in the first sample. DCC are to be considered epidemiological niches with a high potential for the spread of pathogenic microorganisms.

Nasopharyngeal carriage of several bacterial species such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* is very common in young children (16). These organisms are responsible for most episodes of acute otitis media (4), and nasopharyngeal colonization precedes the development of otitis (10, 11). Day care center (DCC) attendance has been reported as a major risk factor for increased rates of carriage of these respiratory bacterial pathogens (1), for increased incidence of upper respiratory tract infections (3), and for increased prevalence of antibiotic resistance, especially in *S. pneumoniae* (6, 15, 18).

These increased carriage rates in children attending DCCs are thought to be due to increased exposure to multiple strains (27). Certain clones of *S. pneumoniae* are widely disseminated in DCCs (17, 20). It has also been shown that transmission of *H. influenzae* strains occurs within DCCs (24, 28).

For The Netherlands, increased rates of upper respiratory tract infections, implantation of tympanostomy tubes, and use of antibiotics in DCCs have been reported (7). Antibiotic resistance rates among respiratory pathogens in The Netherlands are still quite low, although only limited data from the general population or from patients with meningitis (9, 12) are available. The prevalence of beta-lactamase production among *H. influenzae* isolates from sputum samples taken in The Netherlands was only 6.3% in a recent study (21); these samples originate mainly from elderly patients with chronic pulmonary diseases. No specific data are available for resistance rates among respiratory pathogens in young children.

The aim of the present study was to confirm the hypothesis that children attending day care facilities are exposed more often to potentially pathogenic respiratory bacteria due to close contact to other children, resulting in higher carriage rates and spread of strains within this population. To study the dynamics of colonization, molecular typing methods have been used. The molecular epidemiology of *S. pneumoniae* isolates from this study has been reported separately (4a). Here we report the dynamics of nasopharyngeal colonization by *H. influenzae* as studied by molecular typing methods. The spread of *Moraxella* is currently being investigated.

MATERIALS AND METHODS

Study population. From 4 January through 9 March 1999, a total of 535 Dutch children in two matching groups were enrolled in the study. The index group comprised 259 children, aged 3 to 36 months, who were attending a DCC for at least 3 days a week. Sixteen centers in three regions (the central, southern, and northern parts) of Amsterdam, The Netherlands, participated in the study. The control group comprised 276 children, aged 3 to 36 months, who did not receive any form of group care. These children were recruited at four "well-baby clinics" located in the same regions of Amsterdam as the DCCs. The Ethics Committee of the Municipal Health Service of Amsterdam approved the research protocol. Signed informed consent was obtained from one parent of each participating child.

Study design. Parents of the index children received written information about the study through DCC staff. Parents of control children received information by

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mail. If these children were not receiving any form of group care, and if they had an appointment scheduled at the well-baby clinic during the study period, they were invited to participate.

After written informed consent, demographic information and a medical history were obtained for each child through standardized questionnaires completed by one of the child's parents. Questions addressed the child's place of birth, the native country of the parents, family size, history of breastfeeding, health problems requiring specialist attention or surgery, allergies, infections, asthma and chronic bronchitis, and the use of medication, especially antibiotics. In approximately 10% of the cases, some of the parentally recalled information was unclear or missing, and the child's general practitioner was asked for supplementary information. In each region nasopharyngeal swabs were collected at the DCCs and the well-baby clinics within the same 3-week period. Nearly all DCC children were sampled a second time after 4 weeks. Among DCC children overall participation in the study was 50%, ranging from 30 to 76% per DCC. Parents who did not want their child to participate were asked to complete the questionnaire anonymously, so demographics and complete medical histories were obtained for 82% of the DCC children eligible for enrollment. In the control group, approximately 85% of the children who met the criteria for enrollment participated in the study.

Laboratory procedures. Nasopharyngeal samples were obtained with a Dacron pernasal swab (Medical Wire & Equipment Co., Wiltshire, England). Swabs were transported in Amies transport medium to the Microbiology Laboratory of the Municipal Health Service, Amsterdam, The Netherlands, and plated on sheep blood agar and chocolate agar plates (Oxoid, Wesel, Germany) on the same day. After overnight incubation under a CO_2-enriched atmosphere at 36°C, plates were inspected for growth of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *Neisseria meningitidis*. All isolates were identified by standard microbiological methods (14). For all isolates, susceptibilities to four to nine antibiotics, depending on the species, were determined by the disk diffusion method according to the Guidelines of the Dutch Working Party on Antimicrobial Susceptibility Testing (26). All isolates were stored in glycerol broth at −80°C for further studies.

Isolation of DNA from *H. influenzae*. *H. influenzae* isolates were subcultured on chocolate agar plates. DNA was isolated in a standardized manner from one standard loopful of growth by lysis in a buffer containing guanidine isothiocyanate (5), followed by precipitation in isopropanol and by two wash steps with 70% ethanol. The DNA pellet was resuspended in 1 ml of water and stored at −20°C until further use.

RAPD typing. Random amplified polymorphic DNA (RAPD) analysis was performed as previously described with the following primers: RAPD1 (5′GGT TGGGTGAGAATTGCACG3′), ERIC2 (5′AAGTAAGTGACTGGGGTGAG CG3′), and AP3 (5′GTAGACCCGT3′) (2, 25).

The PCR program started with a denaturation phase of 4 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 25°C, and 2 min at 74°C in a PTC-200 thermocycler (MJ Research). PCR products were separated on a 1% agarose gel and visualized with ethidium bromide; gels were photographed, and images were stored digitally. All the isolates from one DCC were tested in the same PCR run and on the same gel. Patterns were compared visually and assigned a different letter for each different banding pattern; slight differences (one weak band) were ignored.

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed as described previously (19). Briefly, agar plugs were prepared by suspending a loopful of growth from a chocolate agar plate in 100 μl of EET buffer [100 mM EDTA, 10 mM ethylene glycol-bis(b-aminoethyl ether)-*N*,*N*,*N*9,*N*9-tetraacetic acid, 10 mM Tris-HCl (pH 8.0)] and mixing this suspension with a similar volume of molten 1.6% agarose (Incert; FMC Bioproducts, Rockland, Maine). Plugs were then incubated overnight with 1% sodium dodecyl sulfate and proteinase K(1 mg/ml), after which they were washed with Tris-EDTA buffer. DNA was then digested with *Sma*I. DNA fragments were separated on a 1% agarose gel by using the Bio-Rad CHEF mapper (run time, 18 h; 6 V/cm; angle, 60° or −60°; switch interval, 5 to 35 s). The gel was stained with ethidium bromide and photographed under UV illumination. Identical patterns were assigned a letter to designate the type.

Statistical analysis. The data collected were entered into a database and analyzed. To evaluate differences between DCC children and controls, the chi-square test or Fisher's exact test was used for comparison of categorical variables. The Wilcoxon test was used for comparison of continuous variables. *P* values of <0.05 were considered statistically significant.

Crude and adjusted odds ratios (OR) and 95% confidence intervals (95% CI) were calculated by using logistic regression to evaluate differences in health status and bacterial carriage between DCC children and controls.

RESULTS

A total of 535 nasopharyngeal cultures were collected from 535 children aged 3 to 36 months. The 259 children in the DCC group had a higher median age (1.53 versus 0.88 years) and a smaller family size than the 276 controls ($P < 0.001$). Among controls, one or both parents more frequently originated from a foreign country ($P < 0.001$). The two groups of children did not differ in gender, country of birth, whether or not they had been breastfed, or duration of breastfeeding.

Medical history. Comparison of the two groups showed that DCC children had been ill about 5 times more often during the past 2 months (Table 1); coughing and rhinitis were the most frequent symptoms. The groups showed no difference in the incidence of earache or ear discharge.

An interesting difference between the two groups was the increased use of antibiotics in the DCC group (18%) versus the control group (9%) in the preceding 2 months (penicillins, 79%; macrolides, 16%; others, 5%).

Bacterial carriage and susceptibility to antibiotics. Nasopharyngeal carriage rates for *M. catarrhalis*, *S. pneumoniae*, and *H. influenzae* were two to more than four times higher in the DCC group. After adjustment of the odds ratios for age, family size, and a history of asthma or chronic bronchitis, the differences remained significant.

H. influenzae was isolated from 37% of the DCC children and from 11% of the control children (adjusted OR, 4.30); the corresponding figures for *M. catarrhalis* were 80 and 48% (adjusted OR, 4.55) and those for *S. pneumoniae* were 58 and 37% (adjusted OR, 2.51). The percentages of colonized children in the second sample were 37, 81, and 64% for *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*, respectively. Serotyping showed four strains of type b in DCC children and one strain of type b in a control child ($P = 0.20$ by Fisher's exact test). All five children carrying *H. influenzae* type b had been vaccinated with *H. influenzae* type b vaccine. No increased antibiotic resistance was found in DCC isolates compared to isolates from the control group. Only 1 isolate (0.24%) of *S. pneumoniae* in a child from the control group showed decreased susceptibility to penicillin; resistance to macrolides in this species was found in 9 of 424 isolates (2.1%), with no difference between DCC children and controls. Beta-lactamase-production was found in 91% of *Moraxella* isolates, again with no difference between DCC children and controls. Decreased susceptibility of *H. influenzae* to amoxicillin was found in 11 of 225 isolates, 4 of which produced beta-lactamase; 10 of these strains were from the DCC group, but the difference did not reach statistical significance.

Evaluation of typing methods for *H. influenzae*. Twenty-four strains from two DCCs and 11 strains from the control group were initially selected in order to compare PFGE with RAPD analysis. With PFGE 27 different patterns were obtained. RAPD with a single primer was less discriminatory, but by using three different primers (ERIC2, RAPD1, and AP3) the same discriminatory power could be obtained as with PFGE. Isolates showing identical PFGE-types were also similar by RAPD typing. Because RAPD typing is technically more simple and equipment was available in our laboratory, all isolates were then typed with RAPD using these three primers. Strains were compared only to other strains from the same DCC. All

TABLE 1. Health status of children attending a DCC compared to that of children cared for at home

Health status	No. (%) of children		Crude OR[c] (95% CI)	Adjusted OR[c,d] (95% CI)
	DCC[a] (n = 259)	Controls[b] (n = 276)		
Illness, past 2 mo	226 (87)	162 (59)	4.82* (3.11–7.46)	5.43* (3.34–8.81)
Coughing	204 (90)	111 (69)	4.26* (2.46–7.39)	5.13* (2.80–9.42)
Rhinitis	211 (93)	128 (79)	3.74* (1.96–7.13)	4.41* (2.18–8.94)
Fever	151 (67)	88 (54)	1.69* (1.12–2.56)	1.77* (1.12–2.78)
Diarrhea	93 (41)	45 (28)	1.82* (1.18–2.81)	1.65* (1.03–2.64)
Vomiting	69 (31)	41 (25)	1.30 (0.82–2.04)	1.28 (0.78–2.10)
Skin rash	51 (23)	22 (14)	1.85* (1.07–3.21)	1.97* (1.09–3.57)
Ear ache	39 (17)	21 (13)	1.40 (0.79–2.49)	1.41 (0.76–2.63)
Ear discharge	16 (7)	6 (4)	1.98 (0.76–5.18)	2.41 (0.86–6.74)
GP[e] visit in past 2 mo[f]	123 (54)	87 (54)	1.03 (0.69–1.54)	1.15 (0.74–1.78)
Medication in past 2 mo[f]	88 (39)	67 (41)	0.90 (0.60–1.36)	0.95 (0.61–1.49)
Antibiotics in past 2 mo[f]	46 (18)	26 (9)	2.08* (1.24–3.47)	2.35* (1.33–4.16)
Antibiotic use during sampling	2 (1)	2 (1)	1.07 (0.15–7.60)	1.45 (0.17–12.17)
Medication daily	39 (15)	31 (11)	1.40 (0.85–2.32)	1.35 (0.77–2.35)
Allergies	36 (14)	40 (15)	0.95 (0.59–1.55)	0.93 (0.54–1.59)
Asthma or bronchitis	34 (13)	9 (3)	4.48* (2.11–9.55)	3.67* (1.62–8.32)
Specialist visit	92 (36)	93 (34)	1.08 (0.76–1.55)	0.90 (0.61–1.34)
ENT surgery[g]	14 (5)	7 (3)	2.20 (0.87–5.53)	1.09 (0.40–2.99)

[a] Children aged 3 to 36 months, attending a DCC at least 3 days a week.
[b] Children aged 3 to 36 months, not attending any form of group care.
[c] *, statistically significant ($P < 0.05$).
[d] Adjusted OR, OR adjusted for age and family size.
[e] GP, general practitioner.
[f] Data available only if parents reported any illness during the past 2 months; n = 226 for DCC children; n = 162 for controls.
[g] ENT, ear, nose, and throat.

isolates from the controls were treated as one group. A cluster was defined as a group of two or more strains with similar genotypes. An example of the RAPD results with one primer for one DCC is shown in Fig. 1.

Clustering of genotypes of *H. influenzae* within DCCs. For molecular typing, 94 of 97 strains isolated from the first sample of children attending DCCs, 93 of 96 strains from the second sample, and 27 of 30 strains from the control group were still available. The remaining nine strains had lost viability during storage at −80°C. Within the 27 isolates from the control group, only 2 isolates (4%) had similar genotypes. Among isolates from DCCs, 37 isolates (39%) from the first sample belonged to a cluster ($P < 0.01$). A similar picture was obtained for the second sample, with 43 isolates (46%) belonging to a cluster. In Table 2 the clustering of isolates within a group or DCC and the sizes of the clusters are shown.

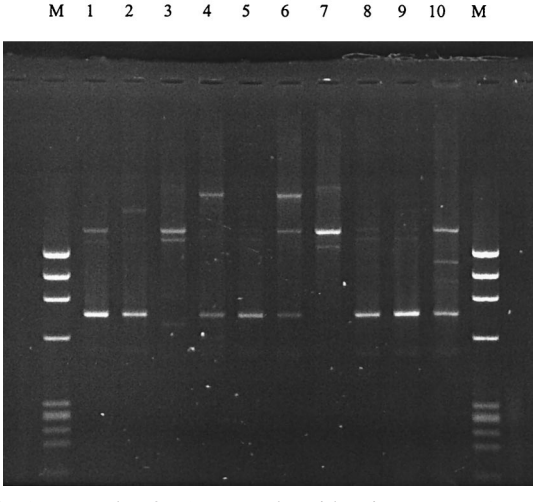

FIG. 1. Example of RAPD results with primer RAPD1. Lane M, molecular weight marker ϕX174/HAEIII. Lanes 1 to 10 represent 10 isolates from one DCC; lanes 5, 8, and 9 show identical patterns.

Dynamics of colonization. To show the dynamics of the colonization of *H. influenzae* isolates within DCCs, Table 3 was constructed. To enable aggregation of results from different DCCs, RAPD types occurring in both sample rounds within one DCC were numbered I to IV in order of descending occurrence. RAPD types occurring more than once but exclusively in one sample round were designated V to VII. RAPD types occurring only once within all samples from a given DCC were designated singletons. Results from both sample rounds, including typing of the isolates, were available for 252 children.

Of the 90 children with a positive culture in the first sample, 47 were not carrying *H. influenzae* in the second sample. Of the 43 children with persistent colonization, only 3 carried the same type in the second sample as in the first, and 13 had acquired an *H. influenzae* type that had been found in another child in the first sampling round.

Of the 162 children who were not colonized in the first sample, 50 were colonized 4 weeks later; in 20 children, again, an *H. influenzae* type was found that had been present in another child in the first sampling round.

TABLE 2. Clustering of genotypes of *H. influenzae*

Group[a]	No. of children	No. of S1[b] isolates	No. of S1 isolates in clusters	S1 cluster size(s)	No. of S2 isolates	No. of S2 isolates in clusters	S2 cluster size(s)
Control	276	30	2	2	NS[b,c]		
DCC_00	7	4	2	2	1	0	
DCC_01	15	7	3	3	3	0	
DCC_02	14	3	0		6	2	2
DCC_03	15	7	3	3	8	4	4
DCC_04	19	3	0		3	0	
DCC_05	16	8	5	3, 2	6	2	2
DCC_06	24	12	8	4, 2, 2	9	5	3, 2
DCC_07	13	2	2	2	7	4	2, 2
DCC_08	12	7	2	2	5	4	2, 2
DCC_09	12	7	6	3, 3	9	7	5, 2
DCC_10	19	4	2	2	6	0	
DCC_11	15	5	0		4	2	2
DCC_12	27	9	0		11	4	2, 2
DCC_13	20	6	2	2	8	7	5, 2
DCC_14	15	8	0		2	2	2
DCC_15	16	5	2	2	8	0	
Total DCC	259	97	37		96	43	

[a] Codes denote individual DCCs.
[b] S1, first sample; S2, second sample.
[c] NS, not sampled.

DISCUSSION

The results of genotyping corroborate the hypothesis that extensive transmission of nasopharyngeal *H. influenzae* occurs in the DCC setting. Around 41% of the *H. influenzae* isolates belonged to a cluster shared by more than one child in both sample rounds. In addition, the fact that 34 to 40% of the children had acquired a (new) strain in the second sample belonging to a genotype that had been found before in the same DCC suggests that colonized children within the same DCC are the source for colonization. Theoretically, the clustering of types and the acquisition of circulating strains could be explained by a common source outside the DCCs. This possibility could not be excluded on the basis of the numbers in Table 3. However, transmission from child to child within the DCC seems to be a much more plausible explanation.

The high turnover of strains is demonstrated by the fact that nearly half of the children lost their strains over a period of 4 weeks. Similar results were found in a study of pharyngeal carriage of *H. influenzae* in 38 children in two DCCs (23). In that study, which also addressed the simultaneous carriage of more than one strain, a median number of 1.4 genetically distinct isolates per culture was found. Carriage rates found in that study were 81% for *H. influenzae* and 48% for *S. pneumoniae*. The differences from our results may be caused by the use of selective media for isolation of *H. influenzae*, the sampling area (throat versus nasopharynx), and the older age of the children. A longer duration of carriage of *H. influenzae* was reported by Yano et al. (28) in a study of children attending a DCC; however, this study involved only six children monitored over a 2-year period and the findings were based on multiple isolations of *H. influenzae* from one child. Also in studies of aboriginal children in Australia, prolonged carriage with one strain of *H. influenzae* over more than 8 months was described; this seems, however, to be related to a special epidemiological setting where impaired immunity might predispose children to prolonged carriage (22).

TABLE 3. Dynamics of colonization in combined DCC cohorts

Colonization status[a] in 1st culture	No. of children with the following colonization status in the 2nd culture:									Total
	I	II	III	IV	V	VI	VII	S	U	
I	2	2		1		2		3	7	17
II	1								4	5
III			1							1
IV									1	1
V	2	1				3		4	9	19
VI										0
VII										0
S	5	1				3	1	11	26	47
U	16	4				4	3	23	112	162
Total	26	8	1	1	0	12	4	41	159	252

[a] Types I to IV, clustered types found in both sampling rounds within a DCC; types V to VII, clustered types found in only one sampling round within a DCC; S, singleton type, occurring only once in a given DCC; U, uncolonized children.

Our study confirmed the findings of other investigators that children attending DCCs carried potentially pathogenic bacteria such as *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in the nasopharynx more often, and had symptoms of upper respiratory tract infections more often, even after correction for the higher median age of children attending day care. We could not confirm an increased incidence of otitis media, though our study may have been too limited in numbers and time to demonstrate such an effect. The increased carriage rates are most probably due to increased exposure resulting from crowding in DCCs; this effect has been suggested previously to explain the higher carriage rates in children with more siblings (27).

Despite a more frequent use of antibiotics we did not find increased antibiotic resistance rates in DCC isolates versus controls. Actually, resistance rates were even lower than those reported in other studies in The Netherlands. This may be due

to the fact that most isolates in those studies originated from elderly people with chronic obstructive pulmonary disease who were being treated with antibiotics more often. Low resistance rates are thought to be related to restricted use of antibiotics in a community. In this study 13.5% of all participating children had used antibiotics in the 2 months preceding the sampling. In several studies abroad, much higher figures are mentioned; for example, De Lencastre et al. reported that 8% of healthy children attending DCCs in Lisbon used antibiotics and an additional 20% did so in the month preceding the study; in their study, 20% beta-lactamase production in *H. influenzae* was found (8). Moulin et al. (13) reported an average of three antibiotic courses per child per year in France and 56% beta-lactamase production. Principi et al. in Italy (16) reported that 25% of the children used one or more courses of antibiotics in a 3-month period and that the rate of beta-lactamase production among *H. influenzae* strains was 5.8%. This suggests a close relation between antibiotic consumption and resistance rates.

The high turnover of respiratory pathogens in DCCs might be an important factor in the spread of resistant clones of these bacteria in the community, as suggested by Sa-Leao et al. (20). This may occur only when resistant strains are already prevalent in a community and high consumption of antibiotics exerts sufficient selective force in subpopulations such as those in DCCs.

In summary, this study describes increased rates of colonization with respiratory bacterial pathogens, and high turnover and transmission rates of *H. influenzae* carried in the nasopharynx, in children attending DCCs. Given similar findings on increased carriage rates in children attending day care in many studies and the results of St. Sauver et al. (23) for a DCC in Michigan, it seems reasonable to assume that these findings apply to DCCs in general. This study also shows that this increased transmission does not per se lead to selection of resistant clones, even though prevalences of illness and antibiotic consumption are higher among children attending day care than among controls. The potential for rapid spread of pathogenic or resistant microorganisms is, however, clearly present in these centers.

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