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Turnover of Nonencapsulated *Haemophilus influenzae* in the Nasopharynges of Otitis-Prone Children

ANDERS SAMUELSON,[1]* ANDERS FREIJD,[2] JON JONASSON,[1] AND ALF A. LINDBERG[1]

Division of Clinical Bacteriology, Department of Immunology, Microbiology, Pathology and Infectious Diseases,[1] *and Division of Oto-Rhino-Laryngology, Department of Clinical Sciences,*[2] *Karolinska Institutet, Huddinge Hospital, Huddinge, Sweden*

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Restriction enzyme analysis of total genomic DNA was applied to study the epidemiology of nontypeable *Haemophilus influenzae* (NTHI) isolated from the nasopharynges of children with recurrent acute otitis media (AOM). The turnover of strains, as judged from genetic fingerprinting of a total of 213 *H. influenzae* isolates collected prospectively during a 2-year study period from 38 children under 3 years of age, was examined in relation to episodes of AOM as well as to courses of antibiotic treatment. The children were selected if they had had at least one episode of AOM before 1 year of age and if more than two nasopharyngeal isolates of *H. influenzae* were recovered. The 213 *H. influenzae* isolates (90% NTHI) recovered corresponded to 128 different DNA fingerprints. Fifty-eight percent of the fingerprints were observed only once, whereas 42% appeared on two or more occasions in isolates from the same individual or in close relatives, i.e., brothers and sisters. Sixty-seven percent of these strains had a minimum colonization period of 2 months or less. Intermittent nasopharyngeal colonization periods longer than 5 months could be demonstrated for 13% of the strains. The present data suggest that intermittent colonization is due to endogenous reinfections. Genetically identical NTHI strains from unrelated individuals were never identified. As expected from the observation of a relatively high proportion of persistent colonizations, no correlation was found between episodes of AOM and the acquisition of new strains of *H. influenzae*, nor was any direct relation between antimicrobial therapy and the elimination of nasopharyngeal colonization with a particular strain of *H. influenzae* observed.

Acute otitis media (AOM) is a common infection in the first years of life. *Streptococcus pneumoniae* together with *Haemophilus influenzae* and, to a lesser extent, *Moraxella catarrhalis* are the major bacterial causes (23). All three species frequently colonize the upper respiratory tracts of children under 4 years of age (5, 11, 13–15, 17, 19). Asymptomatic older children and adults are rarely found to be colonized. Previous studies suggest a direct relationship in otitis media between organisms recovered simultaneously from the nasopharynx and the middle ear (3, 18). The highest frequencies of nasopharyngeal carriage of these potential pathogens are reported in children suffering from clinically evident AOM (6, 7, 22). AOM-prone children tend to be colonized more often than non-AOM-prone children (1, 2, 5–7, 10).

The vast majority of *H. influenzae* isolates from the respiratory tract are nontypeable *H. influenzae* (NTHI) strains. A large pool of different strains seems to be circulating in the community at any one time. NTHI strains are considerably more heterogeneous genetically compared with *H. influenzae* type b strains (20, 21). Epidemiologic studies on the nasopharyngeal carriage of NTHI have been performed with healthy children in day-care centers (25, 26). In their study from Sweden, Trottier et al. (26) reported an average carrier rate of 39% for preschool-age children, with 74% of the children being colonized at some time during the 4 winter months when the study was conducted. The pattern of nasopharyngeal colonization suggested that the younger children acquire and eliminate a number of different strains, some of which are shared with other children, whereas other strains are restricted to a single host (26).

The hypothesis addressed in the present study is that persistent and to some extent cryptic colonization with NTHI is not uncommon in children with recurrent otitis media and may lead to AOM through endogenous reinfection. The reason for examining nasopharyngeal carriage of NTHI instead of concentrating on the middle ear is as follows. Using a fluorescein isothiocyanate-conjugated oligonucleotide probe which specifically hybridizes with the 16S rRNA of *H. influenzae*, we have demonstrated in a recent study of children free of clinical infection and with adenoid hypertrophy that the bacterium, even if it is absent from the nasopharyngeal mucosa, could be found to be sequestered in macrophage-like cells located subepithelially near the crypts in sections of adenoid tissue (8). Therefore, we have speculated that the adenoid could serve as a reservoir for NTHI, causing endogenous reinfections of the nasopharyngeal mucosa (24). This environment would nearly always be the source of the middle ear infection since it is generally accepted that the bacteria responsible for AOM migrate from the nasopharyngeal cavity through the eustachian tube to the middle ear (3, 18).

In the study described in this report, restriction enzyme analysis of total genomic DNA was applied to study the epidemiology of NTHI strains isolated from the nasopharynges of children with recurrent AOM. The turnover of strains, as judged from genetic fingerprinting of a total of 213 *H. influenzae* isolates collected during a 2-year study period from 38 children under 3 years of age, was examined in relation to episodes of AOM as well as to courses of antibiotic treatment. As expected from the observation of a relatively high proportion of persistent colonizations, no correlation was found between episodes of AOM and the acquisition of new strains of *H. influenzae*, nor was there observed any direct relation be-

* Corresponding author. Mailing address: Division of Clinical Bacteriology, F82, Huddinge Hospital, S-141 86 Huddinge, Sweden. Phone: 46-8-746 50 11. Fax: 46-8-779 46 47.

tween antimicrobial therapy and the elimination of nasopharyngeal colonization with the particular strain of *H. influenzae* that was supposed to be the causative agent. The present data also suggest that endogenous reinfections of the nasopharyngeal mucosa might contribute to the increased colonization periods.

MATERIALS AND METHODS

Subjects and study design. From a prospective study of 150 otitis-prone children (10), 38 children were selected on the basis of the following criteria: (i) each child included had had at least one episode of AOM before 12 months of age, and (ii) *H. influenzae* had been isolated on three or more occasions from the nasopharynx of each child. During the first 30 months of life, 32 of these children had six or more episodes of AOM, and 6 children had two to five episodes of AOM. The mean ages of the children when *H. influenzae* was first isolated were 17.6 months (range, 7 to 36 months) for the 25 boys and 20.1 months (range, 6 to 38 months) for the 13 girls. The children were followed from December 1980 to November 1982 by a small group of surgeons at the ear, nose, and throat outpatient clinic at Huddinge Hospital. At each monthly examination, a sample for nasopharyngeal culture was obtained. Illnesses necessitating physical examination between routine visits and antimicrobial drug prescriptions were recorded. Otological examination was performed with a stereoscopic operation microscope. The criteria for normal otoscopic status, AOM, and secretory otitis media have been described earlier (10). The following principles for antibiotic treatment were applied. Primary episodes of AOM were treated with penicillin V (25 mg/kg of body weight per day divided into two dosages for 10 days). Therapeutic failures were treated by using the antibiotic susceptibility test of the previous nasopharyngeal culture as a guide. The most frequently used alternatives following therapeutic failure with penicillin V were amoxicillin (20 mg/kg/day divided into two doses for 10 days), trimethoprim-sufamethoxazole (6 and 30 mg/kg/day, respectively, divided into two doses for 10 days) or erythromycin (50 mg/kg/day divided into three doses for 10 days).

Isolation, cultivation, typing, and storage of bacteria. Nasopharyngeal samples were obtained with a calcium alginate swab (Calgiswab; Inolex, Glenwood, Ill.). The samples were inoculated onto chocolate agar plates (hemoglobin [20 g; BBL], GC agar base [72 g; BBL], IsoVitaleX [20 ml; BBL], 2,000 of ml distilled water) within 3 h after sampling and were incubated in 5% CO_2 in air at 37°C overnight. One of the authors (A.F.) collected the vast majority of the samples and also examined the agar plates after primary cultivation. The satellite phenomenon and the lack of hemolysis on blood agar with *Staphylococcus aureus*, growth requirements for X and V factors (4), and inability to synthesize porphyrins from δ-aminolevulinic acid (16) formed the basis for the diagnosis of *H. influenzae*. The capsular serotype was determined with polyvalent antiserum (Difco, Detroit, Mich.), and if it was positive specific antisera for the capsular types a to f (Wellcome, Beckenham, United Kingdom) were used. The isolates were stored in 1-ml aliquots at −70°C. No attempt was made to subcultivate several different colonies from the primary agar plate for comparative studies. β-Lactamase production was determined with nitrocefin disks (Biodisc, Stockholm, Sweden) after a minimum of subcultivations and before storage at −70°C.

Preparation of DNA. The bacteria were cultivated on chocolate agar and were incubated in humidified air plus 5% CO_2 at 37°C for 24 to 48 h. Half of the bacterial growth on one 100-mm-diameter plate was suspended in 0.4 ml of buffer (50 mM NaCl, 50 mM EDTA, 50 mM Tris hydrochloride [pH 7.5]). The DNA was extracted as described by Loos et al. (18). In short, bacterial cells were lysed with sodium dodecyl sulfate (Sigma) and treated with RNase and proteinase K (Boehringer Mannheim, Mannheim, Germany). This was followed by three extractions with phenol-chloroform-isoamyl alcohol (25:24:1) and three extractions with chloroform-isoamyl alcohol (24:1). DNA was precipitated with isopropyl alcohol. After a short centrifugation the supernatant was discarded and the DNA pellet was dried under vacuum and was finally dissolved in TE (10 mM Tris hydrochloride, 1 mM EDTA [pH 8.0]).

Digestion with restriction enzymes. Purified DNA (5 to 10 μg) was digested with *Eco*RI (Boehringer Mannheim) and, in selected cases, was also digested with *Cla*I (2 U/μg of DNA) at a buffer concentration of 10%, according to the recommendation of the manufacturer.

Electrophoresis. Agarose (0.6%) gel electrophoresis (16 h at 0.7 V/cm) in 0.5 TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) was used. The gel was stained in ethidium bromide and was left to clear in water for 1 h at 22°C before being photographed in UV light.

DNA fingerprinting. The chromosomal DNAs from all 213 *H. influenzae* isolates were extracted and digested with the restriction enzyme *Eco*RI. After agarose gel electrophoresis the banding patterns were compared. The DNA digests representing the isolates from one patient were normally applied onto the same gel in chronological order. The DNA fingerprints (i.e., the gel banding patterns) were classified as either indistinguishable or different. In the majority of cases it was easy to determine if the isolates were identical. If the judgment was uncertain the digestion was repeated with *Cla*I. With this approach it was possible to make a reliable comparison of all 213 isolates. For example, all six isolates from patient 32 were different (Fig. 1, lanes 2 to 7, respectively). Each unique fingerprint was labelled with a two-letter combination and a Roman numeral (I to VI). Figure 1 also shows an example in which isolates with a certain fingerprint reappeared later in the same patient. Eight isolates from patient 26 yielded four fingerprints, I to IV (Fig. 1, lanes 8 to 14, respectively). In this patient, isolates with fingerprint III appeared on two occasions (Fig. 1, lanes 10 and 13), with isolates with other fingerprints being isolated in the interval. In lanes 16 and 17 of Fig. 1 the isolates corresponding to lanes 10 and 13, respectively, were applied next to each other to facilitate the comparison. This procedure was always performed when there was a suspicion that isolates with identical fingerprints were separated by other isolates in the agarose gel. The same methods have been used by other investigators and have been found to be reproducible and reliable (3, 12, 18).

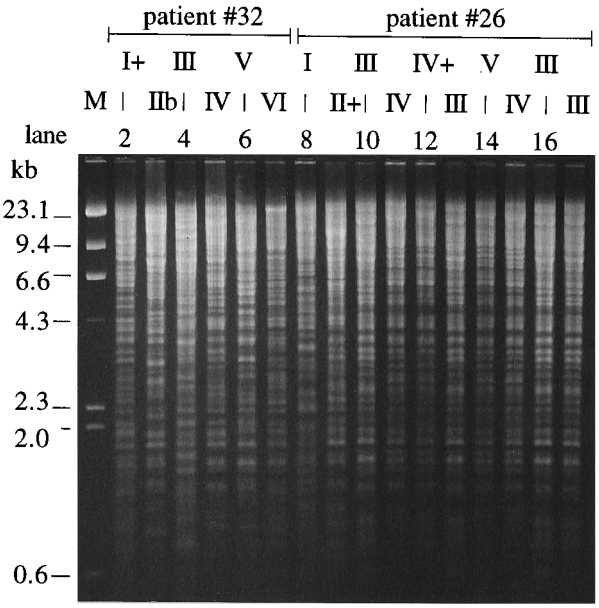

FIG. 1. Total genomic DNA from *H. influenzae* isolates digested with *Eco*RI and then subjected to agarose gel electrophoresis. Patients 32 and 26 were two unrelated children. Lane 1, molecular mass marker; lanes 2 to 7, isolates from patient 32 in chronological order (+ indicates β-lactamase production; b indicates capsular serotype b). Lanes 8 to 15, isolates from patient 26 in chronological order. Lanes 16 and 17, isolates (26-III) from lanes 10 and 13 applied next to each other.

Statistical analysis. χ^2 with continuity correction was used for statistical analysis of the results.

RESULTS

Bacterological findings. Three to 12 *H. influenzae* isolates (total, 213 isolates) were obtained from each child (n = 38) during the study period. The mean observation period was 10.8 months (range, 1.3 to 20 months). The vast majority of the isolates (191 of 213; 90%) were noncapsulated, serologically NTHI. Twenty-two isolates were capsulated: 13 were type b, 2 were type d, 5 were type e, and 2 were type f.

DNA fingerprinting. The 213 *H. influenzae* isolates corresponded to 128 different fingerprints. Fifty-eight percent (74 of 128) of these fingerprints were observed only once, whereas 42% (54 of 128) appeared on two or more occasions. For the latter a minimum colonization period for each particular strain was estimated. Sixty-seven percent (36 of 54) of these strains had a minimum colonization period of 2 months or less (Fig. 2). Nasopharyngeal colonization periods longer than 5 months could be demonstrated for 13.0% (7 of 54%) of the strains. The median colonization period was 5 weeks.

Intrafamilial spread of strains. The patients in the present study were unrelated to each other except for three pairs of siblings: (i) a 7-month-old boy (patient 20) and his 3-year-old brother (patient 36); (ii) twin boys, 12 months old (patients 16

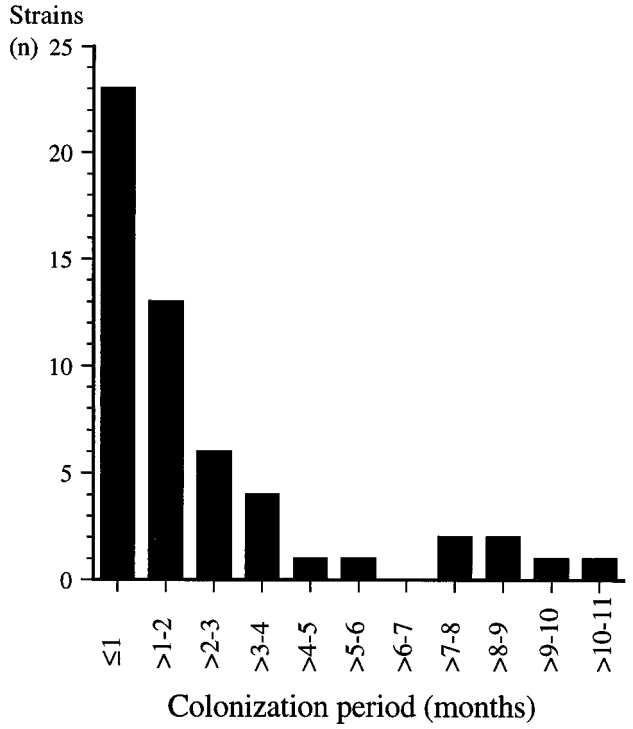

FIG. 2. Minimum colonization period for the 54 *H. influenzae* strains isolated on two or more occasions.

and 17), and (iii) twin girls, 18 months old (patients 29 and 30). On seven different occasions pairwise sharing of the same *H. influenzae* strain was noted (Fig. 3 and 4). The synchrony of the colonizations was also evident for *M. catarrhalis* and *S. pneumoniae* (Fig. 3).

Acquisition and elimination of strains in relation to AOM and antibiotic treatment. Clinical data concerning otomicroscopic findings (AOM, secretory otitis media, normal otological status) and antibiotic treatment collected during the prospective study from 1980 to 1982 (10) were examined in relation to the results of genetic fingerprinting as well as β-lactamase production by and the capsular serotypes of all 213 isolates. The relation between the acquisition and elimination of *H. influenzae* strains and clinical symptoms was very heterogeneous. A long period of nasopharyngeal carriage with the same strain could be accompanied by no episode of AOM as well as several episodes of AOM. Altogether, 34 of 109 instances of AOM (excluding the case of AOM at the time of isolation of the first *H. influenzae* isolate from each patient) were associated with nasopharyngeal colonization with *H. influenzae* as determined in pure culture.

Switches of strains (i.e., fingerprints) occurred both in close relation to AOM and during periods when the children were healthy. The intermittent nature of colonization with *H. influenzae* strains that reappeared on several occasions was noteworthy (data not shown). The relation between AOM and the acquisition of a new *H. influenzae* strain was analyzed on 175 occasions (213 isolates minus the first isolate from each of the 38 patients). There was no significant correlation between the acquisition of a new strain of *H. influenzae* and AOM (χ^2 with continuity correction = 0.09; $P = 0.76$). Similarly, antibiotic treatment of AOM did not result in the clear-cut elimination of the *H. influenzae* nasopharyngeal colonization on the basis of the same 175 observations (χ^2 with continuity correction = 0.02; $P = 0.90$). β-Lactamase production was observed in 13.6% (29 of 213) of the isolates. On 58% of the occasions when a β-lactamase-positive strain was isolated, the patient had been treated with amoxicillin not more than 4 weeks earlier. The corresponding figures were 12.5% for penicillin V, 12.5% for trimethoprim-sulfamethoxazole, and 17% for the patients who had not received any antibiotics. Eight patients were each infected with one strain that on several occasions temporarily produced β-lactamase (data not shown). Finally, it should be noted that in 17 of 31 instances when two *H. influenzae* isolates with the same fingerprint were found with a minimum colonization period of at least 1 month, other pathogens were found between the times of isolation of two isolates at proportions of 46% *S. pneumoniae*, 25% other strains of *H. influenzae*, 19% *M. catarrhalis*, and 10% *Streptococcus pyogenes.*

DISCUSSION

NTHI causes AOM by continuous spread from the nasopharynx to the middle ear cavity through the eustachian tube (18). Thus, understanding the mechanisms of colonization of the nasopharyngeal mucosa is a central issue in the study of middle ear disease. Other pathogens like *S. pneumoniae* and *M. catarrhalis* are frequently found together with *H. influenzae* in the nasopharynx. Therefore, inferences as to the etiology of AOM cannot always be made from nasopharyngeal isolates. In the present study on the turnover of noncapsulated *H. influenzae* in the nasopharynges of otitis-prone children in relation to AOM and antibiotic treatment, no correlation was found between episodes of AOM and the acquisition of new strains of *H. influenzae*. Nor was there observed any direct relation between antimicrobial therapy and the elimination of nasopharyngeal colonization with the particular strain of *H. influenzae* that was supposed to be the causative agent. Several NTHI strains reappeared after being absent from the nasopharynges for up to 11 months (Fig. 2). We have speculated that a strategy by which NTHI accomplishes longstanding, nonsuppurative infections would be to sequester itself intracellularly in lymphoid tissue and thereby become less accessible to the immune defense of the host and to antimicrobial agents (8). If this was so, endogenous reinfection of the nasopharyngeal mucosa might occur in connection with failure of the mucosal immune defense against NTHI (e.g., precipitated by a viral infection).

Groeneveld et al. (12), studying the presence of *H. influenzae* in sputum samples, suggested that there is a coincidence of endogenous or exogenous reinfection by NTHI with exacerbations of chronic obstructive pulmonary disease. Some of these patients were persistently infected with the same NTHI strain for periods of up to 23 months. Groeneveld et al. (12) also noted that antibiotic treatment, although obviously relieving the symptoms, was not effective in eradicating NTHI from patients with chronic obstructive pulmonary disease. In our study, 42% (54 of 128) of the strains represented by a unique DNA fingerprint reappeared on one or several occasions in the same individual, regardless of antimicrobial therapy. Intermittent nasopharyngeal colonization periods from 5 to 11 months could be demonstrated for 13% (7 of 54) of the strains isolated on two or more occasions. The intermittent nature of colonization may reflect a lack of sensitivity of the sampling and culture technique rather than a real loss of the organism. Our earlier study of quantitative bacterial cultures from adenoid lymphatic tissue indicated that when NTHI was found in adenoid cell suspension only 50% of the corresponding nasopharyngeal cultures were positive for the presence of NTHI (9).

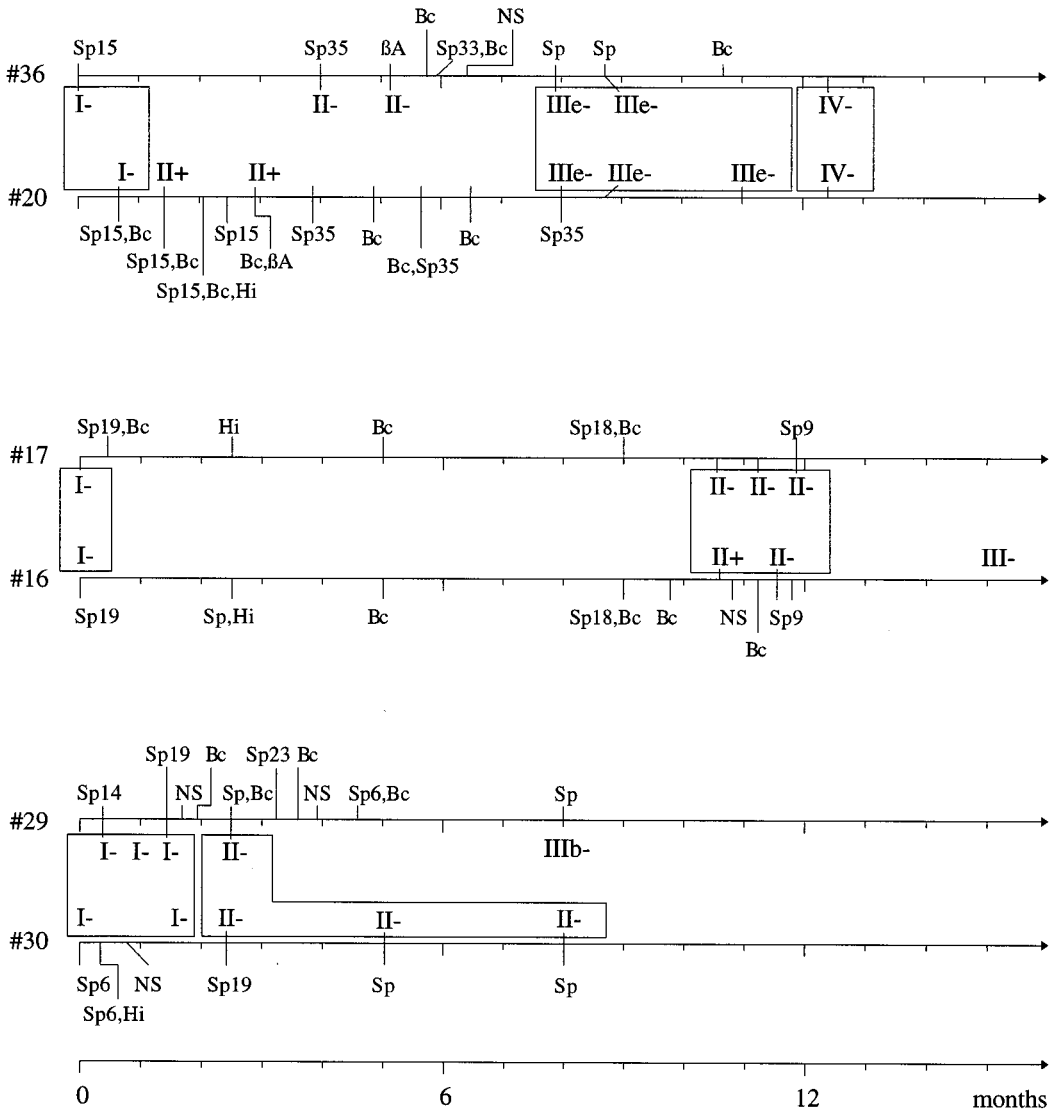

FIG. 3. Chronological chart showing the relation between isolates from three pairs of siblings (patients 20 and 36, patients 16 and 17, and patients 29 and 30). Genetic fingerprints of *H. influenzae* are indicated with Roman numerals starting with I for each patient. The letters after the Roman numerals designate capsular serotype a, b, c, d, e, or f. The plus or minus signs indicate a positive or negative β-lactamase test, respectively. The isolates within the boxes have identical fingerprints. Sp, *S. pneumoniae* (capsular subtype, if determined); βA, *S. pyogenes*; Bc, *M.* (*Branhamella*) *catarrhalis*; Hi, *H. influenzae* (isolate lost); NS, negative sample.

We have postulated that the adenoid could serve as a reservoir for endogenous reinfections (24). The counterargument that the particular strain of *H. influenzae* is harbored exogenously in other colonized family members or playmates seems unattractive for the simple reason that someone else then would have to be permanently colonized. "Ping-pong" infections could not be documented in the three pairs of brothers and sisters that were included in the study (Fig. 3), and coincidences by chance of identical DNA fingerprints in epidemiologically unrelated NTHI isolates seem unlikely in view of the genetic heterogeneity that could be demonstrated between the isolates from the different patients in the study.

Our theory of endogenous reinfections is further supported by the fact that in 17 of 31 instances when two *H. influenzae* isolates with the same fingerprint were found with a minimum colonization period of at least 1 month, other pathogens were found between the times of isolation of two isolates at proportions of 46% *S. pneumoniae*, 25% other strains of *H. influenzae*, 19% *M. catarrhalis*, and 10% *S. pyogenes*. With respect to the low number of cases, these proportions closely follow the proportions of these pathogens that are usually reported in the literature for isolates recovered from the nasopharynges and also from middle ear aspirations from randomly selected patients with AOM (6, 7, 22, 23). Therefore, it seems likely that only a minor proportion of the instances of AOM could be deemed to be caused by the colonizing strain of *H. influenzae*, i.e., those cases (34 of 109; 31% [data not shown]) in which *H. influenzae* was isolated in pure culture in connection with AOM. In view of this and the relatively high proportion of persistent colonizations, it seems less surprising that no correlation was found between changes of the *H. influenzae* strains causing infection and AOM. The lack of any proved effect of the antimicrobial drugs that were used on the *H. influenzae* colonization pattern could be taken as additional evidence for the theory of intermittent nasopharyngeal colonization because of endogenous reinfections.

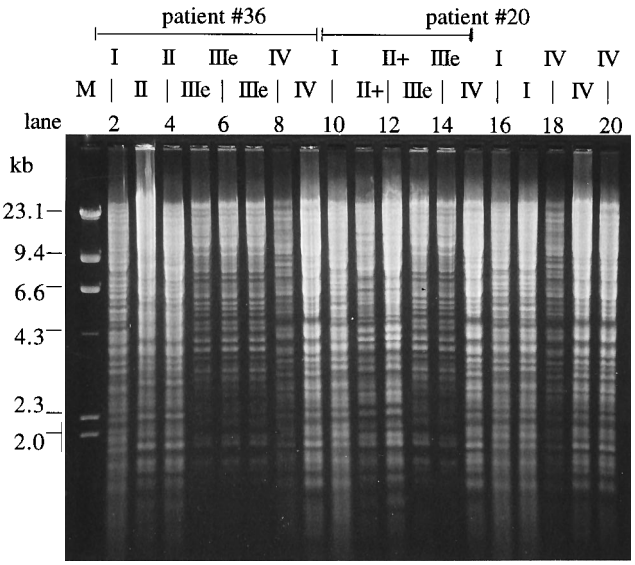

FIG. 4. Total genomic DNA digested with *Eco*RI after agarose gel electrophoresis. Patient 20 and 36 are a 7-month-old boy and his 3-year-old brother. Lane 1, molecular mass marker; lanes 2 to 9, isolates from patient 36 in chronological order; lanes 10 to 15, isolates from patient 20 in chronological order. Lane 16, 20-I; lane 17, 36-I; lane 18, 36-IV (same isolate as in lane 8); lane 19, 36-IV (same isolate as in lane 9); lane 20, 20-IV. Indistinguishable isolates; 36-I compared with 20-I, 36-IIIe compared with 20-IIIe and 36-IV compared with 20-IV.

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