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Determination of the Epidemiology and Transmission of Nontypeable *Haemophilus influenzae* in Children with Otitis Media by Comparison of Total Genomic DNA Restriction Fingerprints

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It is assumed that the causative bacteria in children suffering from otitis media reach the middle ear via the eustachian tube. The purpose of this investigation was to use endonuclease restriction of bacterial chromosomal DNA to compare isolates of nontypeable (NT) *Haemophilus influenzae* obtained from the nasopharynx and from middle ear (ME) effusions of patients with otitis media. Strains of NT *H. influenzae* were isolated from the nasopharynx (NP) and affected ME from a group of 13 unrelated children with otitis media with effusion (OME). For 12 of these children, identical strains were isolated from the NP and ME in a first episode of OME. Each of these 12 sets differed from the other 11. Six of these children suffered from a second episode of OME with NT *H. influenzae*. Five of these children with recurrence again had identical NP and ME strains. These results suggest that at the time of an episode of OME, there is one predominant strain of NT *H. influenzae* that colonizes both the NP and ME. The strains of NT *H. influenzae* isolated from all six of the second episodes were different from strains from the first episode, indicating turnover of the predominant strain in the NT *H. influenzae* population between episodes. When we investigated three siblings with concurrent episodes of OME, we found that they shared several similar strains of NT *H. influenzae*, thereby demonstrating that within a family, transmission of NT *H. influenzae* from child to child is possible. These results from DNA fingerprinting were essentially identical when compared with results from outer membrane protein subtyping performed on the same set of strains. The analysis of endonuclease restriction patterns of total genomic DNA provides a sensitive measure of genetic dissimilarity between strains and represents an easily applicable method for epidemiological and transmission studies of bacterial infections associated with NT *H. influenzae*.

Otitis media with effusion (OME) is a common disease in children. By 3 years of age, 71% of children have had at least one episode of OME, while one-third of 3-year-olds have suffered from recurrent episodes (31). The majority of middle ear (ME) infections are associated with the presence of certain microorganisms. On the basis of cultures of ME fluid samples, *Haemophilus influenzae* is a common cause of OME (8, 14, 16, 20, 29, 30, 32). The vast majority of *H. influenzae* isolates from the middle ear are serologically nontypeable (NT) (5, 12). NT *H. influenzae* has also been implicated in several systemic human infections, including meningitis, pneumonia, and invasive urogenital infections, indicating that NT *H. influenzae* has significant pathogenic potential (for a review, see reference 23).

Individual strains of NT *H. influenzae* have been characterized by biotyping (on the basis of presence or absence of biochemical properties), serotyping (on the basis of outer membrane protein [OMP] preparations), and subtyping (on the basis of OMP profiles or lipooligosaccharides profiles) (3, 4, 6, 22, 25), and epidemiological studies on paired nasopharyngeal (NP)-ME isolates of NT *H. influenzae* obtained during episodes of OME have been performed by using these approaches (4, 24). Data obtained from these studies indicate that the bacteria isolated from the ME fluid in children with OME have originated from the commensal NP microflora (16, 20, 24) by spreading to the ME through the eustachian tube. Eustachian tube dysfunction (5) or a viral infection (13) may predispose for such a migration of microorganisms into the ME.

However, expression of a certain phenotype by a strain, which is used for the above described characterizations, is potentially variable, particularly in response to selective pressure exerted by the immune system. Since the extensive genetic diversity in the NT *H. influenzae* population (26) is presumably a reflection of diversity at the level of chromosomal DNA sequence, restriction fragment pattern analysis of total genomic DNA from NT *H. influenzae* isolates could provide an alternative method for fingerprinting strains associated with otitis media. This method is relatively independent of environmental modulation of phenotype. Analysis of the pattern of restriction fragments produced by restriction endonuclease digestion of bacterial chromosomal DNA is now widely used to investigate the epidemiology of bacteria and yeast and appears to be a powerful tool in studying microbial transmission (9, 11, 17–19, 28, 33).

In this paper, we used restriction endonuclease fragment patterns to study the epidemiology and transmission of NT *H. influenzae* in children with OME. Specifically, we studied the relationship between the NP and ME strains, the strains associated with recurrent episodes, and strains isolated from siblings and concurrent episodes. Furthermore, we tested

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TABLE 1. Patient demographics, strains used in this study, and summary of results

Patient	Age at episode	Date of episode (mo-day-yr)	Strain(s) (origin)	NP and ME DNA restriction profiles[a]	NP and ME OMP profiles[b]
A	5 mo	1-16-86	83 (RME) and 84 (NP)	Same	Same
	22 mo	6-16-87	2536 (LME) and 2537 (NP)	Same	Same[c]
B	14 mo	6-13-86	6699 (LME) and 6700 (NP)	Same	Same[c]
	20 mo	12-22-86	9456G (RME) and 9458G (NP)	Same	Same[c]
C	10 mo	3-8-86	5755 (LME) and 5756 (NP)	Same	Same
	1 yr	5-7-86	2626 (LME) and 2627 (NP)	Same	Same
D	5 yr	1-9-86	9289 (RME) and 9290 (NP)	Same	Same
	5.5 yr	7-24-86	1174 (RME)[d] and 1175 (NP)	Different	Different
E	15 mo	1-9-86	9274 (LME) and 9275 (NP)	Same	Same
	19 mo	5-13-86	3266 (LME) and 3267 (NP)	Same	Same
F	2 yr	1-30-86	1749 (RME) and 1750 (NP)	Same	Same
G	10 mo	8-6-86	2845 (NP) and 2846 (RME)	Same	Same
H	4 mo	5-23-85	4504 (LME) and 4505 (NP)	Same	Same
I	9 yr	5-23-85	4555 (LME) and 4556 (NP)	Same	Same
J	9 mo	6-5-86	5716 (RME)[e] and 5717 (NP)[f]	Different	Different
K	4 mo	7-10-86	9456W (NP), 9459W (LME)	Same	Same
			9458W (RME)	Different	Different
	8 mo	11-7-86	3939 (NP) and 3943 (LME)	Same	Same[c]
L	10 mo	9-11-86	7172 (NP), 7173 (LME), and 7174 (RME)	Same	Same
M	4 mo	11-21-87	4881 (NP), 4882 (RME), and 4883 (LME)	Same	Same[c]
W1[g]	21 mo	1-23-86	954 (NP) and 955 (LME)	Same	Same
	3 yr	6-25-87	3736 (NP) and 3737 (LME)[d]	Different	Different[c]
	3.5 yr	9-3-87	3782 (adenoid)[d]		
W2[g]	3.5 yr	1-23-86	956 (LME) and 957 (NP)	Same	Same
	5 yr	6-25-87	3750 (NP)[d]		
W3[g]	18 mo	6-25-87	3723 (RME)[d], 3724 (LME)[d]	Same	Different[c]
			3722 (NP)	Different	Different[c]

[a] Results of restriction digests of genomic DNA with endonucleases *Cla*I, *Eco*RI, and *Pst*I; isolates from NP and ME gave either identical (same) or nonidentical (different) restriction profiles.

[b] Results of OMP profiles; isolates from NP and ME gave either identical (same) or nonidentical (different) OMP profiles. Analysis was performed either in a previous study (24) or in this study as indicated.

[c] OMP profiles for these strains were determined in this study.

[d] Plasmid-containing strain.

[e] Strain 5716 (RME) was indistinguishable from 6699 (LME) and 6700 (NP) in patient B first episode.

[f] Strain 5717 (RME) was indistinguishable from 4555 (LME) and 4556 (NP) in patient I.

[g] Siblings in family W.

the stability and reproducibility of restriction patterns after multiple laboratory transfers. The results were compared with the epidemiological observations using OMP profiles performed on the same set of NT *H. influenzae* isolates.

(Preliminary results from part of this work were presented at the Research Forum of the Annual Meeting of the American Academy of Otolaryngology–Head and Neck Surgery, Chicago, Ill., 19 to 23 September 1987 [Bernstein et al., Otolaryngol. Head Neck Surg. **100**:200, 1989].)

MATERIALS AND METHODS

Subjects and strains. Sixteen children entered this study on the basis of a clinical diagnosis of OME concomitant with the isolation of NT *H. influenzae* from the NP and/or from one or both ME. Four of these children were affected bilaterally, and in these cases NT *H. influenzae* was obtained from the left and right ME (LME and RME). Three children out of the total of sixteen were siblings which had concurrent episodes of OME (family W), and the other thirteen children (patients A through M) were unrelated. Six of the unrelated children (patients A through E and K) and two of the siblings (W1 and W2) were sampled at a recurrent episode of OME. The ages at the time of each episode are shown in Table 1.

Clinical procedures. Microbiological samples from each child were taken during surgical treatment (tympanocentesis), and NP isolates were recovered from transnasal swab cultures directed at adenoidal tissue (Nasopharyngeal Calcium Alginate Applicator, Pur-Wraps, Hardwood Products Co., Guilford, Me.). These samples were transported and dispersed in sterile brain heart infusion (BHI) broth (Scott Laboratories, Fiskeville, R.I.). Immediately after tympanocentesis, fluid draining from the middle ear was collected by sterile aspiration and mixed with 2 ml of sterile saline. The obtained samples were sent immediately to the clinical laboratory for cultivation (Division of Infectious Diseases, Children's Hospital of Buffalo). After the surgical procedures, the patients were dismissed from the hospital after 6 to 24 h. Postoperative care did not include antibiotic therapy, and the children were routinely seen within 1 month for postoperative checkups.

Laboratory procedures. (i) Isolation and growth conditions. Portions of the obtained NP and ME samples were cultured on commercially prepared 5% sheep blood agar, phenylethyl alcohol blood agar, chocolate agar and MacConkey agar plates (BBL Microbiology Systems, Cockeysville, Md.), and BHI broth. The 5% sheep blood agar, phenylethyl alcohol blood agar, and chocolate agar plates were incubated at 36°C in a 2 to 5% CO_2 atmosphere for 24 to 48 h. The BHI broth and MacConkey agar plates were incubated at 36°C in a normal atmosphere for 24 to 48 h. *H. influenzae* isolates were identified by colony morphology, Gram stains, and growth requirements for X and V factors (X and V strips were from BBL Microbiology Systems). Capsular serotyping and identification of NT *H. influenzae* strains were

determined with the Phadebact Haemophilus Test (Pharmacia Diagnostics, Piscataway, N.J.). The resulting NT *H. influenzae* isolates were stored in BHI broth plus 10% glycerol at −70°C. One colony of NT *H. influenzae* isolated from each site per patient was selected for further study. A total of 52 NT *H. influenzae* isolates were available for this study.

(ii) Preparation of genomic DNA. Total genomic DNA was isolated either from 8-ml liquid cultures (BHI broth) or from cells scraped off approximately 25% of a lawn of cells grown on a chocolate agar plate. Harvested cells were suspended in 0.8 ml of a solution of 50 mM NaCl–50 mM EDTA–50 mM Tris hydrochloride, pH 7.5. After lysis with sodium dodecyl sulfate (1.6% final concentration), RNase A (Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, Md.) was added (final concentration, 3 μg/ml) and the mixture was incubated at 37°C for 90 min to degrade the cellular RNA. The released nucleic acid was deproteinated by addition of proteinase K (final concentration, 100 μg/ml) (Bethesda Research Laboratories, Life Technologies Inc.) and then incubated at 65°C for 2 h. DNA was further purified by three extractions with phenol-chloroform-isoamyl alcohol, followed by three extractions with chloroform-isoamyl alcohol (21). The nucleic acid was recovered by precipitation with isopropyl alcohol, dried, and dissolved in 100 to 200 μl of 10 mM Tris hydrochloride–1 mM EDTA, pH 8.0. Yield was quantitated by measuring the A_{260} (21). The integrity of the DNA as well as the presence of any extrachromosomal elements was assessed by gel electrophoresis on a 0.7% agarose gel using Tris-acetate-EDTA buffer containing 0.5 μg of ethidium bromide per ml (21).

(iii) Restriction digests. Several different restriction endonucleases with six-base, nonredundant recognition sequences were tested under the conditions recommended by the manufacturer (Bethesda Research Laboratories, Life Technologies Inc.) for their abilities to digest the genomic DNA preparations. Enzymes used for this study were selected on the basis of their abilities to restrict most DNA preparations and to give digests that had little, if any, unrestricted high-molecular-weight DNA, which could produce artifactual differences between strains. The total reaction volume of the digests was 25 μl and contained 2 to 4 μg of DNA. The resulting digests were analyzed by gel electrophoresis (100 V for 5.5 h) on a 0.7% agarose gel using Tris-acetate-EDTA buffer containing 0.5 μg of ethidium bromide per ml and then photographed under UV illumination with a Polaroid camera. *Hin*dIII and *Eco*RI restriction fragments of lambda phage DNA were used as size markers.

(iv) OMP subtyping. Preparations of the outer membrane complex of each isolate, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of these complexes, staining with Coomassie brilliant blue, and determination of the OMP subtype have been previously described in detail elsewhere (22, 24).

RESULTS

Analysis of total genomic restriction profiles. Total genomic DNA preparations from NT *H. influenzae* isolates were tested with several different restriction endonucleases for their abilities to produce complete digests. Enzymes used for this study were selected on the basis of their abilities to restrict most DNA preparations and to give digests that had little, if any, unrestricted high-molecular-weight DNA, which could produce artifactual differences between strains (data not shown). On the basis of these criteria, we selected *Cla*I, *Eco*RI, and *Pst*I restriction endonucleases for use in this study. The results of this study are summarized in Table 1. Only the results from the *Eco*RI digests are shown in the figures. However, for all experiments, identical results were obtained with *Cla*I and *Pst*I endonucleases (results not shown).

(i) Diversity in restriction profiles. Figure 1 shows the results of *Eco*RI digestion of all NP-ME pairs of NT *H. influenzae* obtained from the first episodes of OME in 13 unrelated children (patients A through M). When the restriction fragment patterns of NP isolates were compared for each patient, marked diversity was observed. A similar result was obtained when the ME isolates from each patient were compared. Each child therefore appeared to be colonized with a unique strain of NT *H. influenzae*, with one exception. The ME isolate from patient J was identical to the NP-ME strain of patient I, while the NP isolate of patient J was identical to the NP-ME strain from patient B (data not shown). We can find no trivial explanation to account for these similarities, and as far as we can verify, patient J has no relation whatsoever to patients B or I. However, we cannot exclude possibilities of previous contacts, such as attendance at the same pediatric clinic or close home addresses. All strains found in the children with second episodes of OME again appeared to have unique restriction patterns (Fig. 2A and B).

(ii) Analysis of paired ME and NP NT *H. influenzae* isolates. For each patient in the group of 13 unrelated children, we next compared the restriction pattern produced by the NP isolate with the patterns produced by the corresponding ME isolates obtained from the first episode of OME (Fig. 1A, B, and C). The same pattern for the NP isolate and an isolate from at least one ME was obtained in 12 of 13 children (92% [Table 1]). This identity in pattern of ME and NP isolates within each patient contrasts sharply with the diversity seen between patients, where we found 22 different restriction patterns in the combined first and second episodes of OME for the 13 unrelated children (patients A through M). One child (patient J, Fig. 1B) gave different restriction patterns for the two sites. Another child (patient K, Fig. 1C) had a NT *H. influenzae* isolate from the affected LME which was indistinguishable from the NP isolate. However, the isolate from the affected RME gave a restriction pattern that differed from those of LME and NP isolates. Similarly, for the second episode of OME (patients A through E and K), five of six (83%) of the NP-ME pairs examined gave the same restriction fragment pattern from isolates from both sites (Fig. 2A and B, Table 1). The NP and ME isolates from patient D showed a different pattern.

(iii) Turnover in NT *H. influenzae* populations. To study possible elimination or turnover of the NT *H. influenzae* strains present in the first episode of OME, the NP isolates, representative of the first OME episode, were compared with the paired NP-ME isolates from the second episode. The time between recurrent episodes varied from as little as 2 months up to 17 months. For all patients, the strains present in the second episodes were different from those present in the first episodes, even when the episodes were separated by only 2 months (Fig. 2A and B, Table 1).

(iv) Transmission of NT *H. influenzae* strains between siblings. When we compared isolates from the three siblings (patients W1, W2, and W3), similar restriction profiles of isolates were obtained. Two sisters (patients W1 and W2) had a concurrent first episode of OME (on 23 January 1986), and they had isolates from the NP and the affected ME that gave identical restriction patterns. This suggests that the

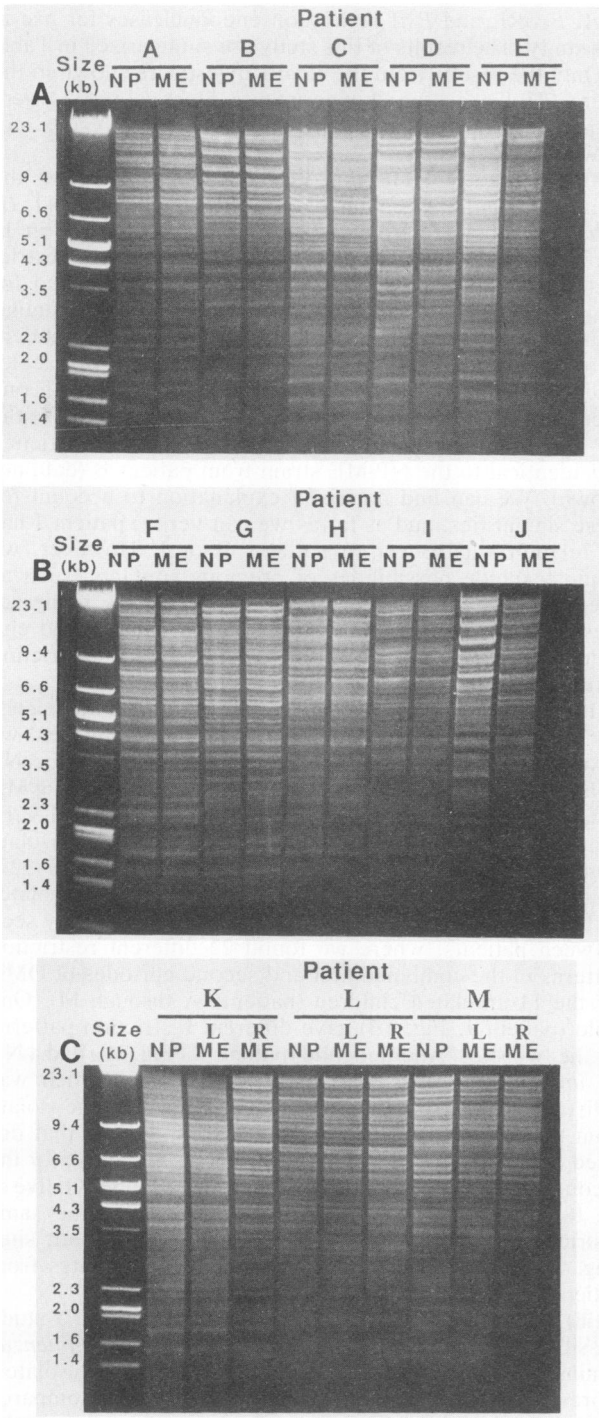

FIG. 1. Restriction endonuclease profiles of paired NP and ME isolates of NT *H. influenzae* from unrelated patients A through M with episodes of OME. Size (kb), Lambda phage DNA size markers in kilobase pairs. L and R, left and right affected ME, respectively.

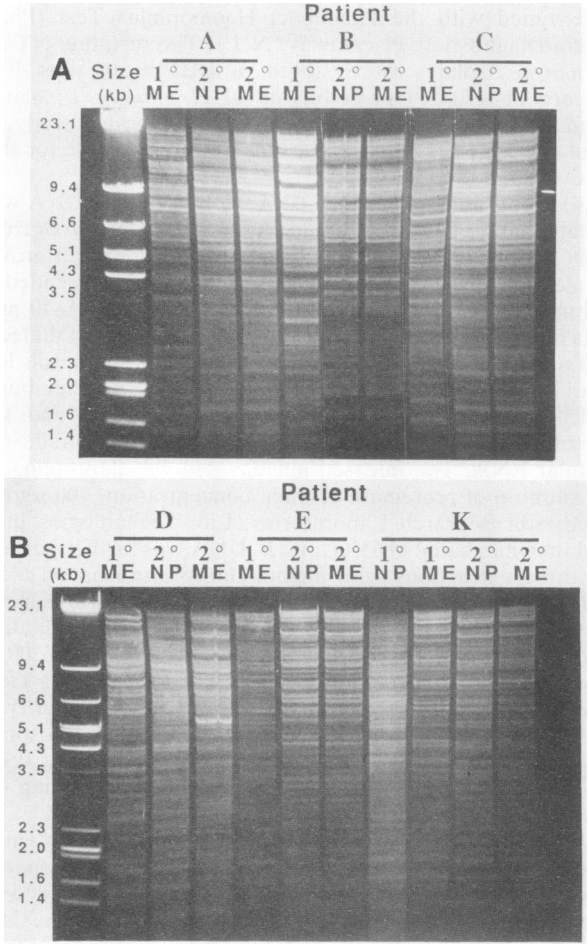

FIG. 2. Restriction endonuclease profiles of first episode (1°) ME isolate and second episode (2°) paired NP and ME isolates of NT *H. influenzae* from patients A, B, C, D, E, and K, who suffered from recurrent OME. Size (kb), Lambda phage DNA size markers in kilobase pairs (kb). L and R, left and right affected ME, respectively.

sisters carried the same NT *H. influenzae* strain in their NP at the same time, and that this strain was also associated with the ME infection in both cases. These two sisters had another concurrent episode of OME (on 25 June 1987), at which time their brother (patient W3) was also affected. This time, a more complex sharing of different strains was found within these patients. The results from this family are summarized in Table 1 and are presented diagrammatically in Fig. 3. In the episode on 25 June 1987, the NP isolate from patient W1 was similar to the NP isolate from patient W3, whereas the LME isolate from patient W1 had a unique restriction pattern and the NP isolate from patient W2 was identical to the RME and LME isolates from patient W3. Interestingly, no NT *H. influenzae* was isolated from the affected ears of patient W2. Finally, in patient W1, the adenoids were removed 3 months after her second episode (on 3 September 1987). We could isolate a NT *H. influenzae* strain similar to the LME isolate from 25 June 1987 but different from the NP strain (data not shown).

(v) Homogeneity of a strain within the NP. As described above, the adenoidal strain from patient W1 isolated on 3 September 1987 appeared to be similar to the LME strain isolated on 25 June 1987 but was different from the NP strain from 25 June 1987. In order to determine whether the NP (i.e., adenoidal tissues) were colonized by one particular strain (one clone) or whether there was coexistence of two or even more different strains, we studied the restriction profiles from 30 isolates taken from the primary isolation plate (adenoid tissue sample from patient W1). Our findings indicated that this patient had a clonal colonization of the

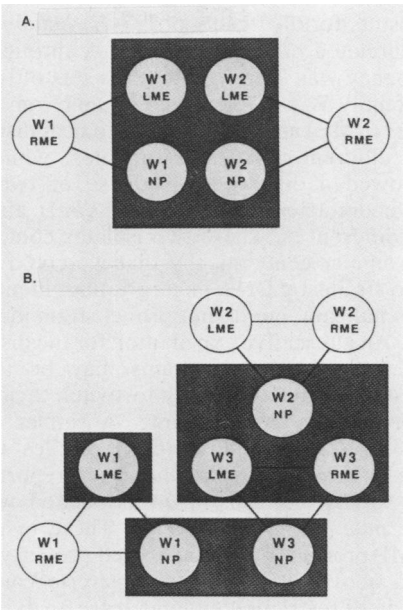

FIG. 3. Concurrent episodes of OME in three siblings of family W. Schematic diagram of NT *H. influenzae* strains found to be similar or dissimilar in patients of family W for episodes on 23 January 1986 (A) and 25 June 1987 (B). Circles within the shaded boxes indicate where NT *H. influenzae* was isolated, and circles outside the boxes indicate unaffected ears or that no NT *H. influenzae* was isolated. All circles within each individual shaded box indicate that identical strains were found. The strain 3782 (adenoid) from patient W1 at 3 September 1987 is not included in this diagram but was similar to this patient's strain 3737 (LME) on 25 June 1987.

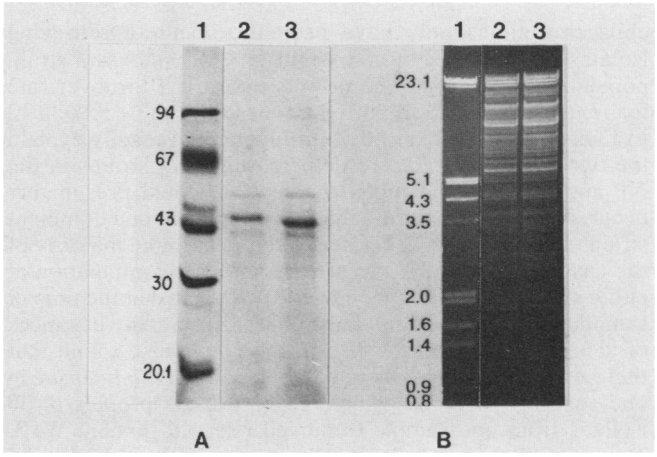

FIG. 4. OMP profiles (A) and DNA fingerprints (B) of two strains with discrepancies in results obtained by using the two techniques. Lanes: 1, molecular weight (in kilodaltons) (A) and DNA size in kilobases (B); 2, strain 3723 (RME); 3, strain 3724 (LME).

adenoid at the time of sampling, that is, there was only one strain present (data not shown).

(vi) Reproducibility of restriction fragment profiles. We also sought to investigate the stability and reproducibility of the restriction digests after repeated transfers in the laboratory. Five isolated colonies were picked from a flame-streaked plate of one randomly selected isolate and each was separately transferred five times. From the five subcultures thus obtained, genomic DNA was again prepared and restriction profiles were compared for similarity with the pattern from the original DNA preparation. Restriction profiles obtained from the five subcultures each transferred five times were identical to the one obtained from the original isolate (data not shown).

(vii) Presence of extrachromosomal elements. Some strains showed the presence of extrachromosomal elements, presumably plasmids (Table 1). We could not find a clear correlation with the presence of these plasmids and any antibiotic resistance (data not shown).

Comparison of restriction fragment profiles with OMP profiles. A detailed description of the results from OMP profile analysis on many of the NT *H. influenzae* strains used in the present study has been reported by Murphy et al. (24). We performed additional OMP profile analyses to complete the OMP data and compared the results obtained in this study from genomic fingerprinting on paired NP-ME NT *H. influenzae* isolates with epidemiological observations obtained by using OMP profiles (Table 1). From Table 1 it can be seen that for the 19 sets of NT *H. influenzae* strains obtained at each OME episode from patients A through M, the NP and ME strains were either the same or different by both techniques. Furthermore, the transmission scheme for the NT *H. influenzae* strains from the siblings in family W for the simultaneous episodes of OME was largely substantiated by the epidemiological results obtained by OMP typing. Therefore, DNA fingerprinting and OMP subtyping gave essentially identical results for strain characterization. Only one discrepancy between the two techniques was observed; isolates 3723 (RME) and 3724 (LME) from patient W3 during the episode on 25 June 1987 were the same as determined by DNA fingerprinting but were found to be different when analyzed by OMP profiling (Fig. 4).

DISCUSSION

Studies on enzyme electrophoretic mobilities have shown considerable genetic diversity in the NT *H. influenzae* bacterial population. Musser et al. (26) found that all of the 65 NT *H. influenzae* strains they examined each had a unique electrophoretic type. It is therefore highly unlikely that two causally unrelated isolates would have the same restriction profile. Consistent with this, isolates of NT *H. influenzae* examined in the present study that were obtained from unrelated children during episodes of OME generally gave restriction fragment patterns that differed from child to child. If siblings in this study are excluded, we found only one case in which two unrelated children had similar isolates. Thus, our observation that in the majority of cases (17 of 19 [89%] of both first and second episodes examined) the NT *H. influenzae* isolate from the ME infection had the same restriction profile as the NP isolate strongly suggests that both isolates represent members of the same clonal population. These results from restriction analysis of genomic DNA of NT *H. influenzae* strains from patients with OME corroborate previous indications that the bacteria in the infected ME are derived from the NT *H. influenzae* population present in the NP (16, 20, 24).

In 2 of the 19 sets of NP-ME isolates from the first and second episodes in patients A through M, we found a different strain in the NP and ME. Since we only examined one isolate from each site, our results do not directly address the possibility that at any one time, a mixture of two or more different NT *H. influenzae* strains is present in the NP and possibly in the derived ME NT *H. influenzae* population. That is, detection of different restriction profiles in these

children could simply have been the result of retrieving isolates of different but coexistent NT *H. influenzae* strain populations present at one or both sites. The turnover rate for *H. influenzae* was reported in one study to be 55% in 12 to 13 days (16). Such a rate of turnover could readily explain the two cases in which we detected different strains in the NP and ME, since a mixed population would presumably occur at some point during the transition from one dominant strain to another. However, it is likely that in the majority of the cases in our study, the NT *H. influenzae* population at either site was relatively homogeneous, at least at the time of sampling, or we would not have observed so many instances of identical strains in the ME and NP. We also found that there was a homogeneous population of NT *H. influenzae* in one case in which we compared restriction profiles of 30 isolates from one sample (removed adenoid [patient W1]). These results are similar to those obtained in studies of pneumococcal otitis media, where simultaneous infection of the ME with two pneumococcal capsular types was observed in only 1% of the infections studied (1).

Examination of restriction profiles at recurrent episodes of OME demonstrated that the NT *H. influenzae* populations present in the NP and ME at the second episode were different from those present at the first, even when the episodes were separated by as little as 2 months. Our observations, as well as those of previous studies (4, 16, 24), therefore provide evidence for turnover in the NT *H. influenzae* population in children with otitis media. Similarly, turnover of strains was found in cases of OME infected with *S. pneumoniae*; recurrent episodes were associated with different capsular types (1). Thus, DNA restriction fingerprinting provides a useful tool for further studies aimed at investigation of NT *H. influenzae* population heterogeneity and turnover in individuals.

Since humans are the only known host for NT *H. influenzae*, the most likely mechanism for acquisition of new strains by individuals is by relatively close contact with others carrying NT *H. influenzae*. One prediction from this hypothesis would be that family contacts would be likely to carry the same NT *H. influenzae* strains at a site at any one time. Our results from examination of restriction profiles of strains isolated from siblings in one family with concurrent episodes indicate that transmission of NT *H. influenzae* can occur between close contacts. The pattern of distribution of strains in these siblings was straightforward for the first concurrent OME episode examined, with all sites carrying identical isolates in both affected children. Although the results from the second concurrent episode examined in these siblings were rather more complex, they are also consistent with a child-child transmission of strains found at both the NP and the ME. Similar to these findings are epidemiological reports on *H. influenzae* type b. There is an association in the biotypes found in secondary contacts of infected individuals, consistent with transmission to contacts (27). Contact with individuals with a *H. influenzae* infection has been proposed as a risk factor for contracting disease. This proposal was based on an apparent clustering of disease outbreaks, consistent with transmission of pathogenic *H. influenzae* strains (2, 7). Close contacts of patients infected with invasive *H. influenzae* type b have been treated with antibiotics to eliminate their carrier status (20, 27).

To further validate our epidemiological observations on NT *H. influenzae* using restriction fragment pattern analysis, we initiated a direct comparison of the results with OMP subtyping, which was performed on the same set of NT *H. influenzae* isolates (Table 1) (24). Both techniques gave identical results for all 19 sets of NT *H. influenzae* isolates from the unrelated children (patients A through M). Only one discrepancy was found. Within the sets of isolates from siblings in family W, we found two strains from patient W3, isolates 3723 (RME) and 3724 (LME), to be indistinguishable with three different restriction enzymes, while the OMP profiles showed a difference. The use of two additional restriction endonucleases (*Xho*I and *Xba*I) also failed to reveal any differences. These two isolates could have been sufficiently similar genotypically that we could not resolve them as two strains by DNA fingerprinting. Identical restriction profiles indicate, but do not prove, strain identity in any single case. An alternative explanation for the discrepancy in results between these two strains may be that NT *H. influenzae* strains have the ability to switch their patterns of OMPs expressed at any one time. A similar observation where two isolates had different OMP profiles while having identical restriction fingerprints has been reported recently for isolates of NT *H. influenzae* associated with chronic obstructive pulmonary disease (10). These results suggest that the OMP profile may not be a fixed property of a strain.

As noted above, one needs to exercise caution in the interpretation of restriction endonuclease analysis results; it is a sensitive technique to detect differences between strains, but identical restriction profiles only strongly suggest, but do not prove, strain identity. In this study, we defined that all differences observed in the restriction profile indicated different strains, regardless of the molecular origin of the restriction site differences. It is likely that most of these differences are due to natural diversity in DNA sequence in the NT *H. influenzae* population, just as this diversity gives rise to multiple electrophoretic types (26), although the presence or absence of plasmids, duplications or deletions of regions of DNA, or phage or transposon insertion may give rise to different restriction patterns in otherwise isogenic strains. However, we feel that until the contribution of these molecular events to pathogenicity in NT *H. influenzae* has been determined, it is prudent to score as different strains those isolates that differ in these characteristics, even if they may originate from otherwise genetically identical strains. On the basis of our data and those of others it appears that instances of such complications in the data are rare.

The technique of restriction fragment fingerprinting represents in general a straightforward approach to investigate the ecology of NT *H. influenzae* populations as well as for longitudinal and cross-sectional epidemiological studies. Technically, it requires approximately the same amount of time and skill as OMP profile analysis. Similarities and differences between strains were easily noticeable on the original Polaroid pictures. Furthermore, restriction endonuclease fragment patterns were stable and reproducible and proved to be a sensitive measure of genetic difference between strains.

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