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Lipopolysaccharide Subtypes of *Haemophilus influenzae* Type b from an Outbreak of Invasive Disease

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Thirty isolates of *Haemophilus influenzae* type b were obtained during an outbreak of invasive *H. influenzae* type b disease and were classified by the electrophoretic profile of their lipopolysaccharide (LPS). The LPS was extracted by a rapid micromethod and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. The isolates could be divided into 1 of 14 subtypes based on the profile of two to four bands. No subtype was predominant. However, all isolates obtained from duplicate sites of the same individual were of the same subtype. Isolates obtained from two patients (6 weeks apart) who attended the same day-care center differed in LPS subtype but were identical in their major outer membrane protein electrophoretic profile. Nasopharyngeal cultures were obtained from healthy children, their immediate families, and employees of the day-care center. Of 13 *H. influenzae* isolates examined from these contacts, only 1 was type b, which was obtained from a day-care worker and had the same LPS subtype and major outer membrane protein electrophoretic profile as one of the disease isolates. The remaining nasopharyngeal isolates were untypable, and most, but not all, were different in LPS pattern. Thus, LPS subtyping of *H. influenzae* type b may be useful in examining the predominance or transmission of a strain during an outbreak and may distinguish some strains not differentiated by outer membrane protein pattern.

Haemophilus influenzae type b is a common cause of bacterial meningitis and invasive disease in infants and young children (18). The potential for spread of *H. influenzae* type b to contacts of individuals with disease has been reported previously (3, 10). All *H. influenzae* type b strains share an antigenically common, serotype-specific capsule. Thus, individual strains cannot be distinguished by serotyping, which makes epidemiological studies by this method difficult. Although *H. influenzae* type b strains may be classified by biochemical characteristics, most isolates causing meningitis are of the same biotype (12). *H. influenzae* type b strains have been subtyped, however, by the electrophoretic profile of their major outer membrane proteins (OMPs) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4, 14).

OMP subtyping has been useful in monitoring the transmission and occurrence of *H. influenzae* type b strains during an outbreak or within a closed community. This methodology has demonstrated that the same subtype may be responsible for recurrent episodes of invasive disease (8), may be isolated from different sites of the same individual (14), and may (or may not) be responsible for a large percentage of cases during an outbreak (3, 14). Although the major OMP profile of each subtype has been reported to be stable and reproducible (4, 14), variations in protein patterns may occur in the same strain when protein-enriched outer membrances are extracted with different detergents or by different techniques (4, 20; T. L. Stull, K. D. Mack, and C. B. Wilson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, K197, p. 209) and when the cells are harvested at different phases of growth (14) and may depend on how much the samples are heated before gel loading (15, 20). Thus, OMP patterns of the same strain may differ when analyzed by different investigators.

Recently, a subtyping system has been developed to classify *H. influenzae* type b strains by the electrophoretic profile of their lipopolysaccharide (LPS) (11). The LPS is extracted by a simple rapid isolation micromethod (RIM) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. Subtyping is based on the mobility of two to four LPS bands that vary among different strains. The profiles are stable under all conditions examined whether or not samples are heated or reduced or whether the same strain is grown in various media to different phases of growth. The method is sensitive and can differentiate some *H. influenzae* type b strains with identical major OMP profiles (11). Isolates with an identical LPS subtype and a different OMP subtype have not been found.

For this investigation, LPS subtyping was used to determine strain variation among isolates obtained during a county-wide outbreak of invasive *H. influenzae* type b disease and to ascertain whether transmission of disease strains occurred at a day-care center with two cases of *H. influenzae* type b infection. In addition, the OMP profile of some isolates was examined for comparative purposes.

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MATERIALS AND METHODS

Bacterial isolates and growth conditions. All *H. influenzae* type b disease isolates used in this study were obtained from patients at Rochester General Hospital, Rochester, N.Y., or Strong Memorial Hospital, Rochester, N.Y. The body site, date of isolation, patient identification, and the associated disease are listed in Table 1. *H. influenzae* isolates were identified by morphology, Gram stain, and hemin (X) and

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TABLE 1. Characteristics of *H. influenzae* type b disease isolates obtained during an outbreak in Rochester, N.Y.

Isolate	LPS subtype	Zone profile	Date of isolation (mo/day/yr)	Child	Site of isolation	Associated disease
R1	BL 12	2,4,8	2/2/82	A	Blood	Meningitis
R4[a]	BL 12	2,4,8	3/7/82	B	CSF[b]	Meningitis
R13	BL 12	2,4,8	3/22/82	C	Blood	Meningitis
R14	BL 12	2,4,8	3/29/82	D	CSF	Meningitis
R2	BL 13	0,1,3,7	2/5/82	E	NP[c]	Meningitis
R3	BL 14	2,8	2/19/82	F	Blood	Meningitis
R6	BL 15	4,10	3/12/82	G	CSF	Meningitis
R11	BL 15	4,10	3/12/82	G	Blood	Meningitis
R12	BL 16	4,6,8	3/17/82	H	Blood	Meningitis
R10	BL 1[d]	2,6,8	3/17/82	I	Blood	Meningitis
R7	BL 1[d]	2,6,8	3/17/82	I	CSF	Meningitis
S2	BL 1[d]	2,6,8	1/9/82	J	Blood	Pneumonia
S16	BL 1[d]	2,6,8	4/3/82	K	Blood	Periorbital cellulitis
R8	BL 17	1,5,7	3/18/82	L	CSF	Meningitis
S11	BL 17	1,5,7	2/14/82	M	Blood	Epiglottitis
S1	BL 18	3,4,6	1/2/82	N	Blood	Periorbital cellulitis
S3	BL 19	3,7	1/12/82	O	Blood	Pneumonia
S12	BL 3[d]	2,4,6,9	3/4/82	P	Blood	Periorbital cellulitis
R9[a]	BL 20	1,3,6,7	3/15/82	Q	Blood	Meningitis
R5[a]	BL 20	1,3,6,7	3/15/82	Q	CSF	Meningitis
S4[a]	BL 20	1,3,6,7	1/17/82	R	Blood	Septic knee
S5[a]	BL 20	1,3,6,7	1/17/82	R	Knee	Septic knee
S10	BL 20	1,3,6,7	1/27/82	S	Blood	Meningitis
S9[a]	BL 21	2,7	1/25/82	T	Blood	Periorbital cellulitis
S8[a]	BL 21	2,7	1/25/82	T	Eye	Periorbital cellulitis
S6	BL 21	2,7	1/24/82	U	CSF	Meningitis
S7	BL 21	2,7	1/24/82	U	Blood	Meningitis
S13	BL 22	3,5,7,9	3/3/82	V	CSF	Meningitis
S14	BL 22	3,5,7,9	3/3/82	V	Blood	Meningitis
S15	BL 23	1,3,6	4/3/82	W	Blood	Epiglottitis

[a] Isolate was obtained from a child attending a day-care center (children Q and T attended the same day-care center).
[b] CSF, Cerebrospinal fluid.
[c] NP, Nasopharynx.
[d] These isolates were identical to previously characterized subtypes (2).

NAD (V) requirement. Type b encapsulation was determined by a modified radioantigen binding inhibition assay (1). In brief, 25 μl of ^{3}H-labeled purified *H. influenzae* type b capsule (1) was mixed with 25 μl of a study bacterial strain at various concentrations and incubated for 60 min. Serum (25 μl) with known anticapsular antibody content was then added, and the percent inhibition of ^{3}H capsule binding to antibody caused by the bacteria was compared with that of known concentrations of nonradioactive purified capsular material to determine the amount of capsule per bacterium (1).

All isolates were grown at 37°C with vigorous shaking in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with factors X and V (2). Bacteria were grown for at least four generations to 10^9 CFU/ml (determined spectrophotometrically) before extraction of LPS or protein-enriched outer membranes.

Extraction of LPS. Details of the RIM extraction procedure have been previously described (11). Briefly, 2×10^9 CFU of bacteria were washed once in phosphate-buffered saline (pH 7.2) containing 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ and resuspended in 300 μl of distilled water. An equal volume of hot (68°C) 90% phenol was added, and the mixture was stirred vigorously at 68°C. The mixture was chilled to 10°C, the phenol-water phases were separated by centrifugation, and the aqueous phase was removed. Three hundred microliters of distilled water was added to the phenol phase, and the extraction was repeated. The aqueous phases were pooled and made to 0.5 M in NaCl, and 10 volumes of 95% ethanol was added. After cooling to −20°C, the insoluble, crude LPS was sedimented by centrifugation and solubilized in 100 μl of distilled water, and the precipitation was repeated. The LPS was resolubilized in 50 μl of distilled water and stored at −20°C.

Isolation of outer membranes. Preparations of outer membranes were obtained by Triton X-100 extraction of cell envelopes, as described by Loeb and Smith (14).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The discontinuous gel system of Laemmli, with minor modification, was used for OMP and LPS analysis (13). For LPS samples, 2 M urea was incorporated into a 15% separating gel. For proteins, a 10% separating gel without urea was used. LPS samples were solubilized, electrophoresed, and silver stained as described by Tsai and Frasch (19). Proteins were electrophoresed and stained with Coomassie blue as described by Laemmli (13).

LPS subtyping. *H. influenzae* type b isolates were classified into subtypes by the electrophoretic profile of their LPS (11). Briefly, each of the two to four LPS bands from each isolate was assigned to one of 12 hypothetical, equidistant zones (the 10 zones previously described were expanded to 12 to include new subtypes). Zones were assigned to LPS bands of previously characterized *H. influenzae* type b strains (e.g., Eag, zones 2, 6, 8; and Mad, zones 2, 4, 6, and 9) (11). Once zone profiles were established, bands from LPSs of unknown isolates were assigned to zones by measuring the distance of a band from the dye front in compari-

son with the distance of a control LPS band from the dye front.

Due to the small amount of material in RIM extracts, the quantity of LPS applied to gels could not be determined. Therefore, a preliminary gel was run containing 5 μl of each RIM extract, followed by a run with a second gel in which the volume of extract was adjusted to obtain optimal resolution of all bands; 3 to 10 μl of RIM extract was usually sufficient. On rare occasions an isolate was encountered from which relatively little LPS could be extracted or from which the LPS stained poorly. LPS bands were faint from these isolates even when the amount of extract applied was tripled (e.g., Fig. 1, lane 5).

Epidemiology. The average annual incidence of *H. influenzae* type b meningitis in Monroe County, N.Y., between 1977 and 1981 was 14 cases per year (range, 10 to 18 cases per year) (S. R. Redmond and M. E. Pichichero, submitted for publication). The number of cases rose to 31 per year in 1982, which was due in large part to a cluster of 14 cases between November 1982 and January 1983. During this same 4-month interval, 9 cases of invasive *H. influenzae* type b disease other than meningitis were identified, bringing the total number of cases in this outbreak to 23.

The patients involved in this outbreak ranged in age from 5 weeks to 4 years. In no child was the infection fatal. Of the 23 cases, 4 occurred in children attending three different day-care centers. Two day-care centers had one case each, and one day-care center (designated KK) experienced two cases separated by a 6-week interval. An epidemiological investigation by telephone interview failed to reveal any contact between the 23 cases during this outbreak other than the two cases from the KK day-care center.

During a 7-day period after identification of the second *H. influenzae* type b infection at the KK day-care center, nasopharyngeal cultures were obtained from family members, day-care center attendees, and staff at the KK day-care center to ascertain whether *H. influenzae* type b colonization had occurred. Nasopharyngeal cultures were screened for *H. influenzae* and type b encapsulation as described above.

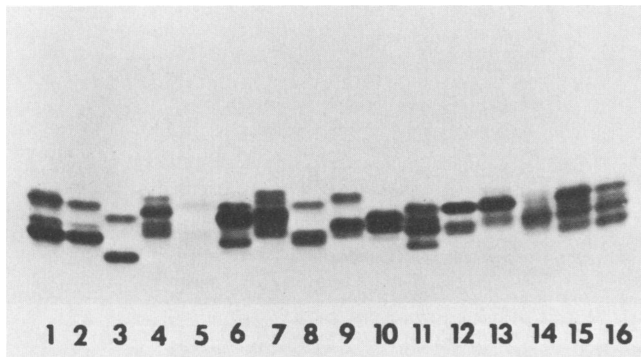

FIG. 1. Electrophoretic profile of LPS from representative subtypes isolated during the Rochester, N.Y., outbreak. LPS was isolated by the RIM. Lanes 1 and 16 contain LPS from previously characterized control strains Eag (BL 1) and Mad (BL 3), respectively. Isolates and subtypes are as follows in the indicated lanes: lane 1, Eag, BL 1; lane 2, R7, BL 1; lane 3, R6, BL 15; lane 4, R9, BL 20; lane 5, S8, BL 21; lane 6, R1, BL 12; lane 7, R2, BL 13; lane 8, R3, BL 14; lane 9, R8, BL 17; lane 10, R12, BL 16; lane 11, S13, BL 22; lane 12, S3, BL 19; lane 13, S15, BL 23; lane 14, S1, BL 18; lane 15, S12, BL 3; lane 16, Mad, BL 3.

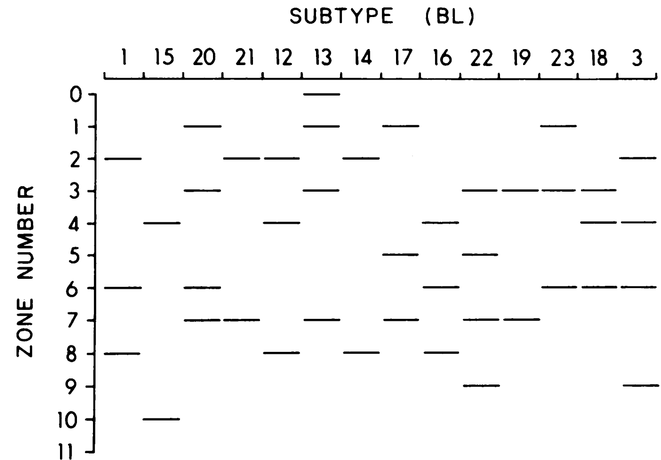

FIG. 2. Diagramatic LPS electrophoretic zone profile of each subtype identified in this study. Bands were assigned to equidistant imaginary zones according to their distance from the dye front in comparison with previously characterized LPS bands (11). Subtypes are arranged in the order as described in the legend to Fig. 1.

RESULTS

LPS subtypes of disease isolates. A total of 30 isolates were obtained from various sites of 23 patients (7 isolates were obtained from a second body site of 7 patients) with the following associated diseases: meningitis (14 patients), periorbital cellulitis (4 patients), pneumonia (2 patients), epiglottitis (2 patients), and a septic knee (1 patient) (Table 1). The LPS profile of each representative LPS subtype identified in this study is shown in Fig. 1 (lanes 2–15), as is that of two strains previously characterized (Fig. 1; lane 1, Eag; lane 16, Mad) (11). The zones occupied by the LPS bands of each subtype are diagrammed in Fig. 2. The LPS subtype and zone profile of each isolate is shown in Table 1. No subtype predominated in this outbreak. However, identical subtypes were found in different children with no known association with each other: BL 12, four children; BL 1, three children; BL 17, two children; BL 20, three children; and BL 21, two children. Isolates obtained from different body sites of the same individual were always identical.

Five isolates fell into two previously characterized LPS subtypes (BL 1 and BL 3) (11); four isolates (two of which were from the same patient) were of subtype BL 1 (represented by isolate R7; Fig. 1, lane 2), and one isolate was of subtype BL 3 (Fig. 1, lane 15). The LPS profile of the other isolates have not been previously identified, thereby increasing the total number of LPS subtypes to 23. In addition, the number of zones was increased from 10 to 12 (zones 0 to 11) to accommodate the position of all the bands currently identified.

Of the 23 patients with invasive *H. influenzae* type b disease, 4 had attended three different day-care centers (Table 1), and 2 patients had attended the same day-care center (KK) (Fig. 1, lanes 4 and 5). The children from the KK day-care center were the only patients known to have had close association with each other. However, the LPS profiles of these isolates were very different, indicating that the children were infected with different strains. Increasing the amount of LPS extract from isolate S8 (Fig. 1, lane 5) did not substantially increase the intensity of the bands, as explained above.

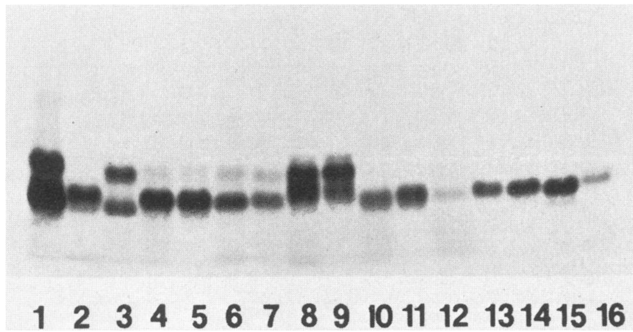

FIG. 3. Electrophoretic profile of LPS from nasopharyngeal isolates. Cultures were obtained from ca. 36 attendees, their families, and staff of a day-care center with two cases of invasive *H. influenzae* type b disease. A total of 13 cultures were positive for *H. influenzae*; 1 was type b and the others were untypable. The LPS pattern of the type b isolate was identical to that of an isolate from a child with meningitis (lanes 8 and 9, respectively). Isolates or sources of isolates are as indicated in the following lanes: lane 1, Eag; lane 2, C 83 (untypable) (11); lane 3, attendee; lanes 4 and 5, mother and her attendee child, respectively; lane 6, attendee; lane 7, attendee; lane 8, type b strain from employee; lane 9, R9, isolated from blood of patient Q; lanes 10 and 11, mother and father of attendee child, respectively; lanes 12 and 13, mother and her attendee child, respectively; lanes 14 and 15, attendee's sibling and mother, respectively; lane 16, attendee's sibling.

Analysis of LPS from nasopharyngeal carrier isolates. Nasopharyngeal cultures were obtained from contacts of the two KK day-care patients to determine whether there were any carriers of type b disease isolates (LPS subtypes BL 20 and BL 21). Of approximately 39 cultures obtained from attendees, siblings, parents, and staff, 13 were positive for *H. influenzae*; of these, one, isolated from a day-care worker, was type b. The other isolates were untypable; their LPS profile is shown in Fig. 3. Strain Eag (Fig. 3, lane 1) and a previously analyzed (11) untypable strain (Fig. 3, lane 2) are shown for comparison.

The LPS profile of the *H. influenzae* type b isolate from the day-care worker (Fig. 3, lane 8) was identical to the LPS profile of the meningitis isolate (BL 20) from child Q (Fig. 3, lane 9). Although the bands in lane 8 (Fig. 3) are stained more heavily than those in lane 9, the LPS profile of each isolate was clearly identical from examination of the gel. Thus, LPS subtype BL 20 may have been transmitted between the infected child and the day-care worker. LPS subtyping is less useful for nontypable *H. influenzae*, because only one or two LPS bands can usually be demonstrated. There was clearly a lack of similarity, however, in the LPS profile of most of the nasopharyngeal isolates (Fig. 3). The LPS pattern from an isolate shown in lane 3 (Fig. 3) was very different from the LPS patterns of other isolates. The LPS patterns shown in lanes 4 and 5 (Fig. 3) were indistinguishable; these isolates were obtained from a mother and her attendee child, respectively. The LPS profile of isolates in lanes 6 and 7 (Fig. 3) differed slightly in the migration of the uppermost band (obtained from day-care center attendees). Isolates from related individuals (Fig. 3, lanes 10 and 11, 12 and 13, and 14 and 15) were also different from each other in LPS pattern. However, three isolates from individuals with no association with each other were identical in LPS pattern (Fig. 3, lanes 13, 14, and 16).

OMP profile of selected *H. influenzae* type b disease isolates. Protein-enriched outer membranes were extracted and analyzed from some *H. influenzae* type b isolates for comparison to their LPS profile. The protein profiles of isolates R7 (BL 1), S8 (BL 27), R9 (BL 26), and NP12 (BL 26) are shown in Fig. 4. Less protein was recovered in the R9 extract, making the bands of this strain appear less intense. However, the major OMP pattern of isolates S8, R9, and NP12 was the same. Thus, this method of subtyping did not clearly differentiate the two disease isolates that were very different by LPS subtyping. Isolate R7 (from an unrelated case of *H. influenzae* type b disease) differed only in that one major OMP band (molecular weight, ca. 35,000) migrated slightly further than the same band from the other strains. The LPS profile of isolate R7, however, is very different from that of the other three isolates. Therefore, for some strains, differences in LPS patterns may be distinguished more easily than differences in OMP patterns.

DISCUSSION

In this report, the use of LPS subtyping to examine the occurrence and transmission of strains during an outbreak of invasive *H. influenzae* type b disease is described. The conventional phenol-water extraction and purification procedure for LPS is very time consuming and not practical for comparison of LPS from many isolates. However, less than 1 μg of LPS is sufficient for visualization of rough LPS in polyacrylamide gels by silver staining (19). Therefore, a rapid micromethod for extracting LPS from many isolates simultaneously has been used specifically for this purpose. Very little equipment is required for this extraction procedure, only LPS appears in the gels, and the electrophoretic profiles are reproducible (11). In addition, the LPS electrophoretic profile remains stable after repeated in vitro subcul-

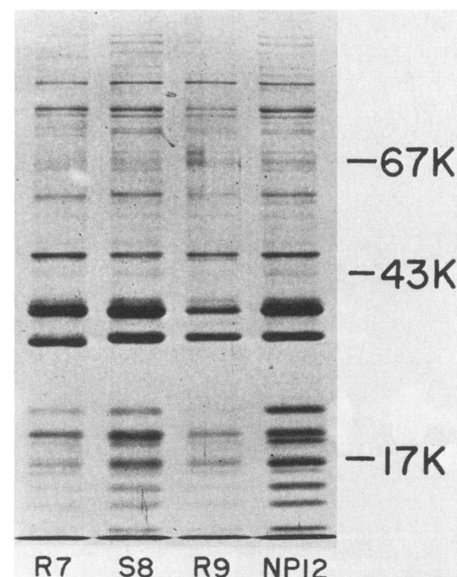

FIG. 4. OMP electrophoretic profile of selected *H. influenzae* type b disease isolates. Protein-enriched outer membranes were prepared by sonication and Triton X-100 extraction and electrophoresed as described previously (14). Isolates S8 and R9 were obtained from children attending the same day-care center. Isolate NP12 was obtained from the nasopharynx of an employee of the same day-care center. R7 was isolated from an infected child who did not attend a day-care center. Molecular weights (times 1,000) are indicated to the right of the gel.

turing or passage of a strain through infant rats (unpublished data). Isolates are subtyped by the profile of their LPS bands in comparison to the LPS bands of control strains. Because some of the bands run relatively close to each other, resolution of the bands is important to this subtyping scheme. Resolution is enhanced by using distilled, rather than deionized, water to make up all reagents (unpublished data) and by the addition of urea to the separating gel (7).

The most common disease that occurred during this outbreak was meningitis, followed by cellulitis, epiglottitis, pneumonia, and septic knee. The small number of isolates in any one subtype precludes association of a subtype with a particular disease. Multiple isolates obtained from different sites of the same child always yielded the same subtype. This result is in agreement with a previous report (14). No one subtype appeared to predominate in this study, which correlates with a study by Loeb and Smith, who examined the OMP composition of isolates during an outbreak in the same community (14). Barenkamp et al. (3, 5) and Granoff et al. (9) have reported that one OMP subtype (1H) predominated in cases of *H. influenzae* type b disease across the United States and in secondary spread of disease in day-care centers. For this study, it is possible that the lack of a predominant LPS subtype may be due to the nature of the population examined, lack of a sufficient number of isolates, or the fact that some isolates of identical OMP subtype differ in LPS subtype.

That LPS subtyping of *H. influenzae* type b can differentiate some isolates with identical OMP patterns has previously been demonstrated (11). In this study, isolates obtained from two infected children attending the same day-care center differed in LPS electrophoretic profile; these isolates were indistinguishable by their major OMP pattern, however, when protein-enriched outer membranes were prepared and analyzed by one procedure (14). Different methods of extraction and electrophoresis of OMPs may produce different protein patterns (4, 20). Therefore, it is possible that the isolates from the two infected children who attended the same day-care center could have been distinguished by a different OMP subtyping system, such as the two-gel system used by Barenkamp et al. (4). Nonetheless, LPS subtyping is highly sensitive and has been used to categorize 63 isolates into 23 subtypes (including isolates obtained from different body sites of the same individual). In comparison, 21 subtypes (9) have been identified from at least 256 isolates by the OMP subtyping system of Barenkamp et al. (5).

Although several children were infected with the same subtype, there was no known association among the children outside of the two children who attended the same day-care center. Thus, healthy individuals acting as a reservoir may have been responsible for transmission of some of the strains (10, 16). Evidence for this hypothesis was found when nasopharyngeal isolates were obtained from contacts of the two infected children attending the same day-care center. Most of the isolates were untypable, which would be expected from a normal population rather than contacts of infected children (18). However, one day-care employee was carrying a type b strain identical in LPS and OMP subtype to that of the isolate from the second infected child. Although it is not possible to determine whether the employee transmitted this organism or obtained it from the patient, it is possible that the employee could serve as a reservoir for transmitting this strain.

It was clear that most of the LPS patterns from untypable *H. influenzae* isolates were different. However, strain identity for untypable *H. influenzae* should be confirmed by OMP subtyping (6, 17), since LPS patterns are less variable than OMP patterns (11). For *H. influenzae* type b disease, however, LPS subtyping is a sensitive and relatively simple procedure for epidemiological investigation.

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