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Outer Membrane Protein and Biotype Analysis of Pathogenic Nontypable *Haemophilus influenzae*

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The techniques of biotype determination and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of outer membrane protein preparations were applied to 35 epidemiologically unrelated isolates of pathogenic nontypable *Haemophilus influenzae*. Three of five isolates obtained from the blood of unrelated newborns with sepsis had concordant major outer membrane protein profiles and were biotype IV. Two of five isolates obtained from the blood of unrelated older children or adults with bacteremia had concordant major outer membrane protein profiles, distinct from the common profile of neonatal strains, and were biotype II. The outer membrane protein profiles of the remaining 5 isolates from blood, 2 isolates from cerebrospinal fluid, and 23 isolates from middle ear aspirates of children with otitis media were unique, although each isolate had peptides with apparent molecular weights of 16,000 and 31,500. These results suggest that a subset of nontypable isolates associated with bacteremia has distinctive strain markers. Their pathogenicity may relate to a predilection for colonizing the female genital tract in the case of the common neonatal strain or an increased ability to evade host defenses.

Haemophilus influenzae isolates are commonly found in the upper respiratory tracts of healthy children and adults (18–20). Most of these organisms are unencapsulated and not typable with antisera specific for the six recognized capsular types (17). Although these organisms rarely cause invasive infection in healthy children and adults, nontypable *H. influenzae* isolates are a frequent cause of otitis media in children (6, 7). They are also commonly isolated in purulent secretions from the lower respiratory tracts of patients with cystic fibrosis (13) and chronic bronchitis (21). Nontypable *H. influenzae* isolates also occasionally cause bacteremic illness in neonates and in older immunocompromised patients (5, 8, 23).

Attempts to understand the epidemiology of diseases caused by nontypable organisms have been hampered by the lack of a useful classification system (22). The biotyping system for *H. influenzae* described by Kilian (9) provides one framework, but its sensitivity to strain variation is limited since only six biotype categories are defined and some biotypes are observed infrequently.

We previously described a subtyping system for *H. influenzae* type b based upon the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of the outer membrane proteins (1). This system was useful in epidemiological studies of type b *Haemophilus* disease (1, 4). In this report, we describe the results of our examination of pathogenic nontypable organisms by similar gel electrophoretic techniques.

MATERIALS AND METHODS

Bacterial strains. Thirty-five epidemiologically unrelated nontypable *H. influenzae* isolates were examined: (i) 23 isolates from middle ear aspirates of children with acute otitis media (isolates kindly provided by Virgil M. Howie, University of Texas School of Medicine, Galveston [17 isolates], and Sarah Long, Temple University School of Medicine, Philadelphia, Pa. [6 isolates]); (ii) 10 isolates from the blood of patients with bacteremia (includes five neonates with sepsis); and (iii) 2 isolates from the cerebrospinal fluid (CSF) of patients with ventriculoperitoneal shunts and meningitis. Five isolates, each related epidemiologically to one of the 35 unrelated strains, were also examined: (i) three isolates obtained from nasopharyngeal specimens of patients whose middle ear aspirations also yielded nontypable *Haemophilus* organisms (provided by Sarah Long); (ii) one isolate from a tracheal aspirate taken on day 18 of life from a neonate with worsening pneumonia; the child had nontypable *H. influenzae* isolated from the blood on day 1 of life and subsequently received a 14-day course of intravenous antibiotics (isolates kindly provided by Peter Krause, University of Connecticut School of Medicine, Farmington); and (iii) one isolate from a CSF sample collected 10 weeks after a previous episode of nontypable *Haemophilus* meningitis in a 15-year-old boy; the patient had a ventriculoperitoneal shunt in place and was suspected of having a cribiform plate abnormality; he had experienced 12 previous episodes

of meningitis (isolates kindly provided by Janet R. Gilsdorf, University of Minnesota School of Medicine, Minneapolis).

H. influenzae isolates were identified by their typical colonial morphology on chocolate agar and their growth requirements for NAD and hemin. Each of the blood or CSF isolates was examined for capsule production within one to four passages of initial isolation. For otitis isolates, the number of passages before examination was unknown. Nontypable *Haemophilus* isolates were defined as organisms which failed to produce iridescent colonies on fresh Levinthal agar (14) and failed to agglutinate strongly with commercial type-specific rabbit antisera prepared against *H. influenzae* types a through f (Burroughs-Wellcome Co., Research Triangle, N.C.). A few isolates showed weak agglutination with several of the antisera or agglutinated spontaneously with saline. Therefore, the blood and CSF isolates were also examined for capsular production by countercurrent immunoelectrophoresis with commercial antisera; all failed to produce precipitin bands (23). Typing of blood and CSF isolates was also confirmed by slide agglutination at the Missouri Division of Health with reference antisera to capsular types a through f provided by the Centers for Disease Control.

Biotype analysis. Biotypes were assigned to isolates by assessing their ability to produce urease, indole, and ornithine decarboxylase in assays modified from those described by Kilian (9, 10). The urease test was performed as described previously (10). Ornithine decarboxylase activity was assayed as described previously (10), with the exception that the Moeller decarboxylase base (Difco Laboratories, Detroit, Mich.) was supplemented with NAD and hemin (Sigma Chemical Co., St. Louis, Mo.), each at 2 μg/ml, and the organisms were allowed to grow for 18 h at 37°C before interpretation of the reactions. The indole test was performed by growing each isolate in tryptic soy broth (Scott Laboratories, Inc., Fiskeville, R.I.) supplemented with NAD and hemin. After 18 h of growth at 37°C, Kovac reagent was added, and the reactions were interpreted as described previously (10).

Outer membrane protein analysis. The methods for preparation of outer membrane derivatives have been described in detail previously (1). In brief, a single colony from an overnight growth on chocolate agar was inoculated into 50 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with NAD and hemin, each at 2 μg/ml. The bacteria were grown to mid-log phase in 250-ml Erlenmeyer flasks at 37°C and 250 rpm in a rotary shaker-incubator (model no. G-25; New Brunswick Scientific Co., Edison, N.J.). Organisms were harvested by centrifugation at 40,000 × *g* for 20 min at 4°C and held frozen at −70°C for up to 2 weeks. Sarcosinate-insoluble membrane fractions were prepared and analyzed by sodium polyacrylamide gel electrophoresis in two gel systems as previously described (1, 12).

RESULTS

Nontypable *H. influenzae* isolates had a limited number of major outer membrane proteins which migrated with apparent molecular weights between 15,000 and 50,000. When isolates were grown in enriched media under defined conditions (1), the gel pattern produced by the outer membrane proteins of each isolate was stable and reproducible. However, significant differences were observed between the gel patterns of different nontypable organisms. The outer membrane protein profiles of seven representative isolates from middle ear aspirates as resolved by the 4 to 24% gradient gel system (Fig. 1A), and the modified Laemmli system (Fig. 1B) are shown in lanes 1 through 7. For comparative purposes, the two most common patterns observed among type b isolates are shown in lanes 8 and 9 (Fig. 1A and B). No two otitis isolates had the same major outer membrane protein pattern, although each isolate had peptides with apparent molecular weights of 16,000 and 31,500.

Figure 2 shows the protein patterns of 10 nontypable isolates obtained from patients with bacteremia. The protein profiles of the invasive isolates were less heterogeneous than those of the otitis isolates. Three isolates recovered from blood specimens of unrelated neonates born over a 15-month period in St. Louis had identical outer membrane protein profiles (Fig. 2A and B, lanes 1 through 3). Isolates from the blood of two other cases of neonatal sepsis had unique outer membrane profiles (Fig. 2A and B, lanes 4 and 5). Two of five isolates obtained from the blood of unrelated older children and adults also had nearly identical major outer membrane protein profiles (Fig. 2A and B, lanes 6 and 7), different from the pattern associated with isolates from neonatal sepsis. With the exception of the five blood isolates with one of the two common patterns, no two epidemiologically unrelated isolates (otitis, blood, or CSF) had concordant outer membrane protein profiles. Furthermore, no nontypable isolate had a protein profile identical with that of any of the type b isolates examined to date (1, 4).

The heterogeneity of the protein profiles of nontypable isolates precluded development of a classification scheme analogous to the one we described for type b organisms. However, examination of the outer membrane protein profiles of epidemiologically related nontypable strains was useful in several clinical settings. The profiles of isolates from the blood and from a tracheal aspirate of a neonate with sepsis and subsequent pneumonia (see above) are compared in Fig. 3A and B, lanes 2A and B. The profiles appear identical on both gel systems. This finding suggests that the child may have relapsed with the same organism responsible for the initial infection. The outer membrane protein profiles of a pair of isolates obtained simultaneously from a nasopharyngeal specimen and a middle ear aspirate of a child with acute otitis

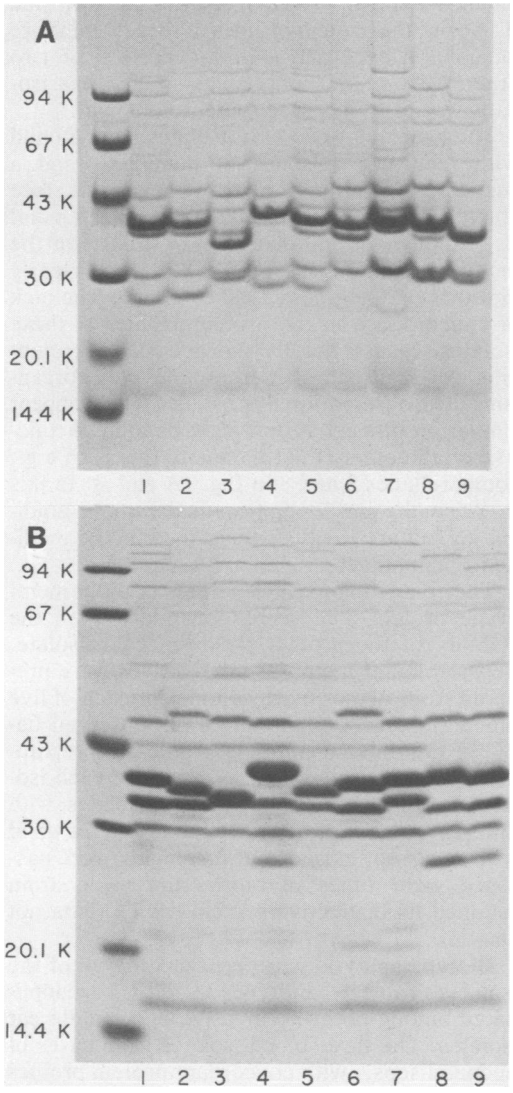

FIG. 1. A, Sodium dodecyl sulfate–4 to 24% polyacrylamide gradient gel electrophoresis of detergent-insoluble outer membrane preparations of seven representative nontypable *H. influenzae* strains isolated from middle ear aspirates of children with acute otitis media (lanes 1 through 7). For comparison, two isolates showing the two most common profiles of type b *H. influenzae* (1, 4) are shown: pattern 1 (lane 8) and pattern 2 (lane 9). The biotypes of the nontypable isolates shown are: I (lane 1), II (lanes 2 and 3), III (lanes 4 and 5), IV (lane 6), V (lane 7). Both type b isolates shown (lanes 8 and 9) are biotype I. Molecular weight standards are α-lactalbumin (14,400), soybean trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000), and phosphorylase b (94,000). B, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Lugtenberg [12] modification of the Laemmli gel system) of the same preparations shown in A. Lanes and molecular weight standards are the same as in A.

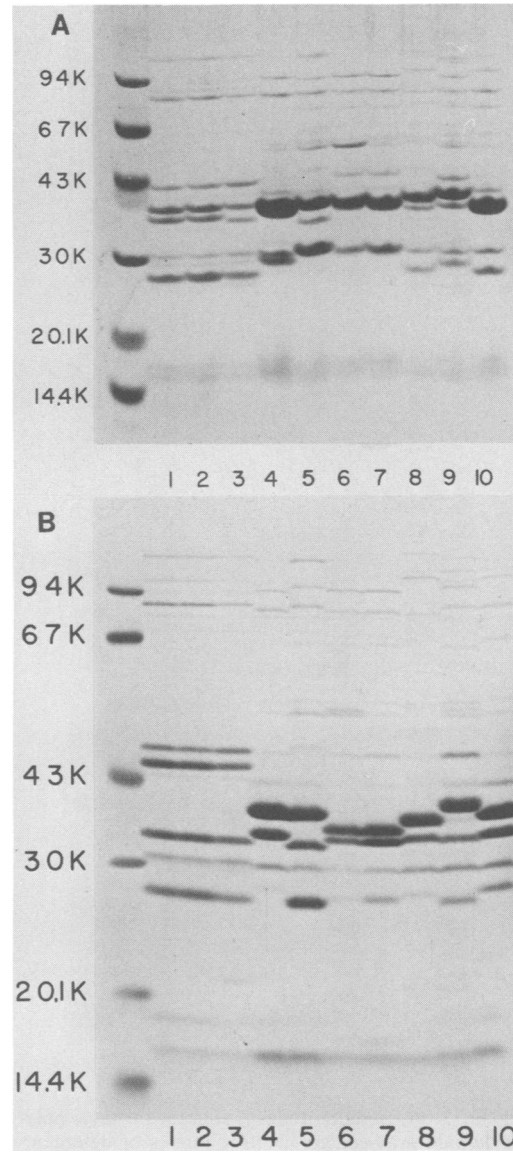

FIG. 2. A, Sodium dodecyl sulfate–4 to 24% polyacrylamide gradient gel electrophoresis of detergent-insoluble outer membrane preparations of 10 nontypable *H. influenzae* isolates from the blood of patients with invasive disease: five neonates with sepsis (lanes 1 through 5) and five immunocompromised older children or adults with bacteremia (lanes 6 through 10). Three isolates from unrelated cases of neonatal sepsis had concordant profiles (lanes 1 through 3). Two isolates from immunocompromised adults with sepsis had concordant profiles (lanes 6 and 7) distinct from the common neonatal pattern. Molecular weight standards are the same as in Fig. 1A. B, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Lugtenberg procedure) of the same preparations shown in A. Lanes are the same as in A, and molecular weight standards are the same as in Fig. 1A.

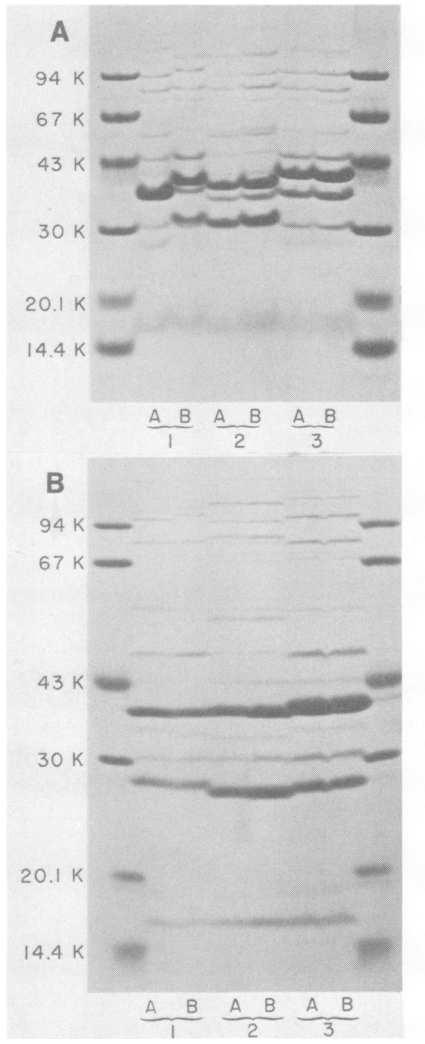

FIG. 3. A, Sodium dodecyl sulfate–4 to 24% polyacrylamide gradient gel electrophoresis of detergent-insoluble outer membrane preparations of three pairs of epidemiologically related nontypable *H. influenzae*: (i) two CSF strains isolated 10 weeks apart from a child with recurrent episodes of meningitis and a suspected cribiform plate abnormality (lanes 1A and 1B); (ii) blood and tracheal aspirate isolates (lanes 2A and 2B, respectively) from a neonate with sepsis and subsequent pneumonia; and (iii) isolates obtained simultaneously from a middle ear aspirate and a nasopharyngeal specimen (lanes 3A and 3B, respectively) from a child with acute otitis media. Molecular weight standards are as in Fig. 1A. B, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Lugtenberg procedure) of the same preparations shown in A. Lanes are the same as A, and molecular weight standards are the same as in Fig. 1A.

media are shown in Fig. 3A and B, lanes 3A and B. Again, the protein profiles of these strains are identical. Concordance of the profiles of two other pairs of nasopharyngeal-middle ear isolates was also observed (data not shown).

The profiles of two CSF isolates from a child with recurrent episodes of meningitis and a suspected cribiform plate abnormality (see above) are shown in Fig. 3A and B, lanes 1A and B. Although the profiles appear similar on the modified Laemmli gel (Fig. 3B), they are clearly different on the gradient gel (Fig. 3A). The lack of concordance in the protein profiles of these isolates suggests that the two episodes of meningitis were caused by different nontypable organisms. The observation that isolates which appear similar on one gel system may be quite distinctive on the other is not unique to this pair, e.g., compare lanes 3 and 8 in Fig. 2A and B. In this instance, the profiles appear similar on the gradient gel but are easily distinguished on the modified Laemmli gel.

The epidemiological usefulness of outer membrane protein analysis is dependent upon the stability of the protein profile of each isolate. Separate outer membrane derivatives were prepared from two or more colonies of each of five blood isolates. Concordance was observed between the outer membrane profiles of preparations derived from different clones of each isolate. Furthermore, the two CSF isolates with different protein profiles obtained from the child with recurrent episodes of meningitis were passaged eight times in broth, and each strain retained its distinctive protein profile (data not shown).

Biotype analysis was performed on all of the isolates (Table 1). Biotypes II and III predominated among both the invasive and middle ear isolates. The three blood isolates from cases of neonatal sepsis with concordant protein profiles were all biotype IV, and the two blood isolates with concordant profiles collected from older individuals with bacteremia were both biotype II. Epidemiologically related pairs of isolates had homologous biotypes, even the two CSF isolates with discordant protein profiles from the child with recurrent meningitis.

DISCUSSION

Early attempts to develop a serological classification system for nonencapsulated *H. influenzae* were hampered by the antigenic diversity demonstrated by these organisms (16, 22). Our results indicate that the outer membrane protein profiles of nontypable *H. influenzae* are also quite variable, precluding development of a classification system based upon these profiles. Nevertheless, all isolates examined had peptides with apparent molecular weights of 16,000 and

TABLE 1. Biotypes of nontypable *H. influenzae*[a]

Biotype	No. of isolates from the following source:	
	Middle ear	Blood or CSF
I	2	0
II	9	6
III	7	3
IV	1	3
V	4	0

[a] Only a single isolate from each epidemiologically related pair was included. All three biotype IV isolates from blood or CSF were obtained from neonates.

31,500 (as do type b isolates). Further studies will determine whether these peptides show immunologic cross-reactivity and are exposed at the cell surface. If so, they could be potentially useful as vaccine candidates for prevention of certain common infections caused by nontypable *H. influenzae* such as otitis media.

Loeb and Smith also found variability in the outer membrane protein composition of 17 nontypable strains isolated from patients seen in Boston (11). They observed that three proteins, designated *e*, *g*, and *h* in their system, had identical mobilities in all nontypable and encapsulated strains examined. The common 16,000- and 31,500-molecular-weight proteins we observed probably correspond to proteins *g* and *e*, respectively, in the Loeb and Smith system, but protein *h* was not observed by us. We also did not observe the additional major outer membrane protein, designated *i*, suggested as being a protein unique to nontypable isolates by Loeb and Smith. Differences in the membrane preparation technique or in the sources of isolates might explain these discrepancies.

The frequencies of the different biotypes among our isolates were similar to those previously reported by Kilian (9). Although the number of blood and CSF isolates examined by us was small, the biotype distribution of this group of strains was similar to the biotype distribution of isolates obtained from middle ear aspirates (Table 1). However, the biotype frequencies of the nontypable strains were significantly different from those previously observed for invasive type b isolates (89% biotype I [3]). The differences between type b and nontypable *Haemophilus* isolates in both their outer membrane protein profiles and their biotypes suggest that nontypable organisms are genetically distinct from type b strains. These results tend to refute the idea that encapsulated type b strains may represent nontypable organisms which have recently become capsule producers (19). (We [1] and Loeb and Smith [11] have previously reported that the spontaneous loss of capsular synthesis among laboratory-passaged type b strains does not result in alteration of their outer membrane protein profiles. An Rd strain derivative, KW 20, and its type b and type d capsular transformants [15] also had identical major outer membrane protein profiles [unpublished observations].) However, we have not yet systematically examined the protein profiles of non-type b encapsulated *H. influenzae* and, therefore, cannot speculate on their possible relationship to nontypable organisms.

The usefulness of outer membrane protein analysis was demonstrated in several clinical situations. Concordance was observed in the protein profiles of isolates obtained simultaneously from nasopharyngeal specimens and middle ear aspirates of three children with acute otitis media. These findings are consistent with the likely pathogenesis of acute otitis media, namely, that nasopharyngeal colonization with a particular bacterium is followed under appropriate conditions by middle ear colonization with the same strain. In another setting, comparison of the outer membrane protein profiles of two CSF isolates from a child with recurrent meningitis suggested that the meningitis episodes were caused by different *Haemophilus* strains. It is important to note that the protein profiles of the two isolates from this child were stable, even after repeated passage in vitro. This observation is similar to our previous findings with type b isolates (1), that is, the protein profiles of type b isolates were found to be stable after passage in vitro or in vivo (infant rats) as well as after natural transmission in humans (1, 2).

We observed that two of five nontypable *H. influenzae* isolates from older children and adults with bacteremia had a distinctive outer membrane protein profile. Furthermore, three of the five nontypable isolates from neonates with sepsis also had a distinctive outer membrane protein profile, different from that of the other pair, and had the relatively rare biotype IV. These findings were intriguing since none of the 30 remaining epidemiologically unrelated nontypable isolates had concordant outer membrane protein profiles. Wallace et al. recently reported that biotype IV strains accounted for 4 of 10 nontypable isolates obtained from the blood of women with obstetrical infections (23). Whether the three isolates with the common outer membrane protein profile from cases of neonatal sepsis in our study bear some relationship to the biotype IV strains described by Wallace has yet to be determined. But it is possible that a biotype IV *Haemophilus* strain with a distinctive outer membrane protein profile has a predilection for colonizing the female genital tract, the likely source from which neonates acquire the infecting organism (5, 8). Alternatively, this

strain and the biotype II strain found in the two older individuals with bacteremia may be more pathogenic than other nontypable organisms.

ADDENDUM IN PROOF

We have recently found that two biotype IV *H. influenzae* strains isolated from neonates in Houston with sepsis (Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 117, 1981) had the same outer membrane protein profile as the common St. Louis neonatal strains.

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