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Outer Membrane Protein Subtypes of *Haemophilus influenzae* Type b Isolates Causing Invasive Disease in Victoria, Australia, from 1988 to 1990

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Outer membrane protein subtyping of 187 isolates of *Haemophilus influenzae* type b (Hib), isolated from children with invasive Hib disease in Victoria, Australia, showed that a single outer membrane protein subtype (1VA) was responsible for 83% of the infections. It was identical to that responsible for the majority of cases of invasive Hib disease in Europe.

Outer membrane protein (OMP) subtyping has been used to epidemiologically link cases of *Haemophilus influenzae* type b (Hib) disease in Europe and the United States. This report describes OMP subtypes from Hib cases in Victoria, Australia, and compares them with those previously reported.

All Hib isolates from patients with invasive disease, admitted to the Royal Children's Hospital in Melbourne, Australia, between February 1988 and February 1990, were stored at −70°C. Cases of invasive Hib disease were defined as those in which Hib was isolated from samples of blood, cerebrospinal fluid (CSF), or other normally sterile sites or from the throats or epiglottides of patients with laryngoscopy-confirmed epiglottitis. Isolates were identified as Hib by colonial morphology, Gram stain, requirement for X and V factors, and agglutination with commercially available polyclonal Hib antiserum (Wellcome Diagnostics, Dartford, United Kingdom).

OMP preparations were performed by a method similar to that of Barenkamp et al. (2), with modifications. Cells were harvested by centrifugation at 3,000 × *g* for 20 min washed, and resuspended in 1.5 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.4). They were frozen (−70°C), thawed, and sonicated on ice. Cellular debris was removed by centrifugation at 3,000 × *g* for 20 min. Cell envelopes were sedimented at 11,600 × *g* for 30 min at 4°C in a microcentrifuge. After extraction with 2% sodium lauryl sarcosinate in HEPES buffer, the detergent-insoluble fraction was washed and recovered by microcentrifugation as described above. The pellets were resuspended in 200 μl of sample buffer containing 63 mM Tris base, 10% glycerol, 2.3% sodium dodecyl sulfonate (SDS), and 5% beta-mercaptoethanol (pH 6.8). They were then heated at 100°C for 5 min and stored at −70°C until required.

OMP preparations were analyzed by using SDS-polyacrylamide gel electrophoresis (PAGE), with a modified Laemmli 11% gel and a 4 to 24% density gradient gel (2). Gels were run with molecular weight standards (Bio-Rad Laboratories, Richmond, Calif.) and known OMP subtypes (provided by D. M. Granoff and L. van Alphen) and stained with Coomassie blue. Samples were coded by number, and their origins were unknown to the observers. Each sample was classified by two observers, and samples of predominant subtypes were sent to L. van Alphen and D. M. Granoff for confirmation.

Isolates were tested for indole, urease, and ornithine decarboxylase production (6). Indole production was tested with a spot indole test by using 1% *p*-dimethylaminocinnamaldehyde (Sigma Chemical Co., St. Louis, Mo.) in 10% HCl; urease and ornithine decarboxylase production were measured in microtiter wells, as described by Tebbutt (11). Biotyping was performed in duplicate and repeated when discordant results were obtained. β-Lactamase production was tested by using a commercially available nitrocephin disk (BBL, Cockeysville, Md.).

Two hundred thirty-five patients with Hib disease were admitted to Royal Children's Hospital during the 2-year study period. Isolates from 187 patients were available for testing, including those from 74 of 84 patients with meningitis, 67 of 94 patients with epiglottitis, and 46 of 60 patients with other Hib diseases. Other infections from which isolates were available, with the number of isolates given in parentheses, included cellulitis (15), pneumonia (13), bacteremia only (11), septic arthritis (5), severe pharyngitis with bacteremia (1) and an infected thyroglossal cyst (1).

A single OMP subtype accounted for 156 (83%) of 187 isolates. It was identical with the predominant European subtype 1—as described by van Alphen et al. (12) and here designated 1VA—which has been reported to be identical with that designated 3L in the system reported by Barenkamp et al. (2). The distribution of OMP subtypes for all cases of meningitis, epiglottitis, and other types of Hib disease can be seen in Table 1. There are no statistically significant differences in subtype distribution by disease (Fisher's exact test). The majority (92%) of all isolates and those in the predominant OMP subtypes (1VA, 95%; 14L, 92%) were biotype 1. Additionally, 14% of isolates were β-lactamase producers.

Three different OMP subtyping methods for Hib have been described previously (2, 7, 13) and have been applied to large numbers of isolates, mostly from the United States and Europe; at least 21 different OMP subtypes are distinguishable and cross-referencing of two methods has been reported (12). In the United States, nearly 90% of isolates are represented by six OMP subtypes (1H, 1L, 2H, 2L, 3L, and 4H),

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TABLE 1. OMP subtypes by Hib disease category

Hib disease (no. of isolates)	No. of OMP subtype isolates[a]						
	1VA (%)	14L	1L	2L	3L	6U	V
Meningitis (74)	59 (80)	6			2	3	4
Epiglottitis (67)	58 (87)	4	1	2			2
Other (46)	39 (85)	2	1	3	1		
Total (187)	156 (83)	12	2	5	3	3	6

[a] Subtype 1VA is identical with the predominant European strains described by Takala et. al. (10); the others are as described by Barenkamp et al. (2). V subtypes are four apparently unique Victorian strains; there were three isolates of strain V1 and one isolate each of strains V2, V3, and V4.

each of which accounts for a substantial minority (10 to 35%) (1, 5). However, in Europe, a single subtype (subtype 1—here designated 1VA) is responsible for 70 to 80% of infections (10, 12); subtype 1 has been reported to be identical to subtype 3L in the American classification (12).

In our laboratory, the predominant subtype, 1VA, was reproducibly different from subtype 3L (Fig. 1), the latter being represented by a small number of our isolates. (The significance of this difference is unknown and will be investigated further by genetic comparison of the strains [8].) The second most common subtype (14L) in this study has not been reported commonly elsewhere. There were small numbers of subtypes 1L, 2L (European subtype 2), and 6U (European subtype 3), and six isolates were unique local isolates, which were designated V (for Victoria) 1 to 4: three isolates were V1, and there was one each of V2 to V4 (see Table 1).

There was no statistical difference, overall, in the distribution of subtypes between disease categories. However, when comparing children younger than 1 year of age with children older than 1 year, subtypes other than 1VA were associated with a larger proportion of cases (27% versus 14%), and this was most significant for meningitis cases (35% versus 13%) (data not shown). Although the differences were not quite statistically significant, the observation is interesting because Takala et al. (10) reported that the relatively uncommon subtype 1c in Finland caused a significantly greater proportion of cases of meningitis (38 of 125, 30%) than of epiglottitis (7 of 80, 9%). It is possible that uncommon subtypes occur more commonly in younger children because of other risk factors, such as day-care center attendance or household crowding, which expose them to a greater variety of Hib strains. This would be consistent with the finding that there is more variation in OMP subtypes (1, 2) in populations (e.g., in the United States) in which the incidence of Hib disease is higher and the average age is lower, presumably because of earlier exposure (3).

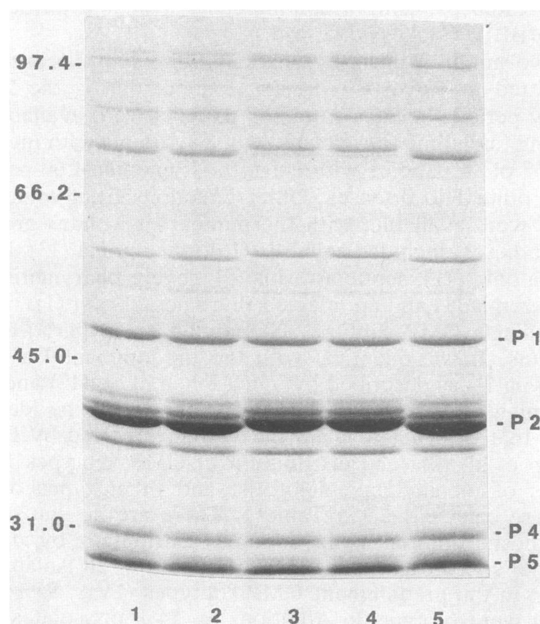

FIG. 1. Density gradient PAGE gel showing two local 1VA strains (lanes 1 and 5), the 1VA strain obtained from L. van Alphen (lane 2), and two 3L strains, one from D. Granoff, St. Louis, Mo. (lane 3), and one from L. van Alphen, Amsterdam, The Netherlands (lane 4). Both strains appear to have identical major OMP P1, P4, and P5 bands, but the P2 band and other minor bands are different. The positions of the molecular weight standards are indicated on the left.

OMP subtyping is a simple but crude method of differentiating strains of Hib. However, it is potentially useful for comparison of isolates from different populations, especially when similarities or differences in subtype distribution appear to correlate with clinical epidemiology. In Australia and Finland, where there is an increased percentage of epiglottitis cases (4, 9) compared with that in the United States (3), there is a relatively similar distribution of OMP subtypes. The true relevance of this finding awaits further study.

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