Differences in subtype distribution of *Haemophilus influenzae* type b from carriers in the general population and patients with meningitis

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Summary. Twenty-four *Haemophilus influenzae* type b strains from 836 children and young adults in an open population were subtyped by outer-membrane-protein (OMP) analysis on sodium dodecyl sulphate-polyacrylamide gels, lipopolysaccharide serotyping and biotyping. The results were compared with those obtained with *H. influenzae* type b strains from 97 patients with meningitis in the same city (Amsterdam). OMP subtype 1 was significantly more common among the CSF isolates than in carrier strains (82% vs 50%; $p < 0.002$). The other OMP subtypes found among carriers were rarely isolated from patients. The lipopolysaccharide serotype and biotype distribution did not differ between the two groups. The combination of OMP subtype 1, lipopolysaccharide 1, biotype I was much more common in isolates from patients than in those from carriers (71% vs 42%; $p < 0.01$). The data suggest that various *H. influenzae* type b subtypes are less virulent than those commonly isolated from invasive infections.

Introduction

More than 95% of systemic *Haemophilus influenzae* infections are caused by type b strains. In The Netherlands, infants under the age of four years are affected mainly with a peak incidence amongst those aged 8–9 months.^{1–3} Although *H. influenzae* is part of the normal nasopharyngeal flora, type b strains are found in only a minority of healthy people (especially young children); colonisation rates vary considerably in open populations.⁴

Person to person spread supposedly occurs by transmission of respiratory tract secretions. Increased *H. influenzae* type b carriage among young household and day-care contacts of patients with *H. influenzae* type b disease correlates with a higher incidence of disease among such contacts.⁵ In the open population carriage rates were reported to be independent of the risk of invasive infection with *H. influenzae* type b.⁶ This discrepancy may be explained by a difference among *H. influenzae* type b strains in their potential to colonise the nasopharynx and their ability to cause systemic disease.⁷

H. influenzae type b is subdivided by analysis of the major outer membrane proteins (OMPs) by sodium dodecyl sulphate-polyacrylamide gel electrophoresis

(SDS-PAGE) (OMP subtyping), lipopolysaccharide (LPS) serotyping and biotyping (BT). OMP subtyping was shown to be highly discriminatory.^{8,9} Hampton *et al.*¹⁰ found that the subtypes isolated from patients with *H. influenzae* type b disease occurred also in carriers. In contrast, strains of OMP subtype 13L were found significantly more often among carriers in an open population than in patients. The numbers in this study were too low to conclude which subtypes of *H. influenzae* type b are typical carriage strains. It was a particular difficulty of this study that a large number of subtypes was found in the USA among isolates from patients with systemic disease.⁹ In The Netherlands, *H. influenzae* type b OMP subtype 1 strains are almost exclusively responsible for cases of haemophilus meningitis,^{1,8,11} and typical carriage subtypes can be identified easily. Therefore, we have compared the subtype distribution of *H. influenzae* type b strains isolated from patients and from carriers in an open population in Amsterdam.

Materials and methods

Study population and collection of samples

Carriage strains were obtained with informed consent. Throat swabs were taken from 492 children aged 4–12 years who attended four City Health Board Centres in Amsterdam for routine vaccinations. Additionally, samples were taken from 196 infants

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aged 0–1 years attending two Infant Health Board Centres and from 148 undergraduate students of medicine and biology aged between 20 and 30 years. All samples were collected between December 1986 and April 1987. Isolates of *H. influenzae* type b from invasive disease were obtained from 97 patients with meningitis who were admitted to hospitals in Amsterdam from 1975 to 1987. A randomly selected series of 80 *H. influenzae* type b strains isolated from patients with meningitis in The Netherlands between 1975 and 1980 and another 96 isolates collected in 1987 were used as reference strains. These strains were collected as part of a nationwide surveillance based on collaboration with the bacteriologists of the Regional Health Laboratories. The submission rate for these meningitis isolates increased from 60% to more than 80% during the first years of the investigation period.¹²

Isolation and typing of *H. influenzae*

Throat swabs were cultured on brain-heart infusion agar supplemented with Levinthal base, NAD 10 mg/L, bacitracin 5 U/ml and with antiserum to *H. influenzae* type b (0.45 ml/10-ml plate) according to the method described by Michaels *et al.*¹³ Antiserum was raised in sheep by the method described previously for raising antisera in rabbits.⁸ The plates were incubated for 36–44 h at 37°C in a humid atmosphere of air with CO₂ 5%. Halo-forming bacteria were selected from all plates. All strains isolated from throat swabs and from CSF were examined for dependence on haemin and NAD for growth, for the inability to convert δ -aminolevulinic acid, and for the presence of a capsule detectable by coagglutination with type specific antisera as described previously.⁸

OMP subtyping was performed by SDS-PAGE of the major OMPs on a linear SDS-polyacrylamide gel after boiling the bacterial cell envelope preparations for 5 min or warming for 30 min at 37°C in SDS 1% as described previously.⁸ A panel of reference strains obtained from Dr D. M. Granoff (St. Louis, USA) and from our own collection were used for comparison.⁹ LPS serotyping was performed with monoclonal antibodies (MAbs) specific for LPS-1 and LPS-10 in a whole cell ELISA as described by Abdillahi and Poolman.¹⁴ Details of the production and characterisation of these MAbs have been published elsewhere.¹⁵ Strains which gave negative results in this test were analysed further by Ouchterlony immunodiffusion with polyclonal LPS-specific antisera as described previously⁸ and elsewhere.¹⁶ Biotyping was performed according to the method developed by Kilian.¹⁷

Statistical analysis

Analysis of differences was performed with the χ^2 test (two-sided, 1df, Yates's correction when necessary), the Odds Ratio (OR), Fisher's exact test (two-sided) and with 95% binomial confidence intervals (CI95).

Results

Carriage of *H. influenzae* type b in Amsterdam

From a total of 836 throat swabs processed, 26 carriers of *H. influenzae* type b were found among 688 children under 12 years (3.8%) but none among the 148 adults tested (table I). The lowest carriage rate of *H. influenzae* type b in the group of children aged under 12 years was in infants under 1 year, the highest in children of 11 and 12 years. The age-distribution of carriage differed strongly from that of meningitis, cases of which are almost entirely restricted to children under 5 years.^{1–3}

Biotypes, OMP subtypes and LPS serotypes of *H. influenzae* type b among patients with meningitis and carriers

Of the 26 carriage strains, 24 were available for further analysis and the results are summarised in table II. Most (19) of the carrier strains were BT I, four strains were BT II and one strain was BT V. The percentage of BT I strains (79%) was not significantly different from the proportion among meningitis strains in Amsterdam (85%).

With OMP subtyping, 12 of the 24 carriage strains were subtype 1; the others belonged to various subtypes which have also been found among strains from patients with meningitis, albeit rarely. This percentage of subtype 1 strains among carriers (50%) was much lower than that among patients with meningitis in Amsterdam, since 80 (82%) of 97 patient strains were subtype 1. This difference is highly significant ($\chi^2 = 9.42$, $p = 0.002$; $OR = 4.71$, $1.64 < OR < 13.67$). As the patient strains were collected between 1975 and 1987 and the carrier strains were collected only in 1987, we considered the possibility that the distribution of subtypes had changed between 1975 and 1987. Therefore, we compared the relative contribution of OMP 1 strains among patient strains in The Netherlands in 1987 with the data on strains isolated between 1975 and 1980 published previously.⁸ The percentage of OMP 1 strains had hardly changed during this period (84% in 1975–1980 and 89% in 1987). The effect of the difference in age distribution between carriers

Table I. *H. influenzae* type b carriers and meningitis cases in The Netherlands

Age group (years)	Number of carriers (%)	Number examined	CI95	Cases of meningitis per 100 000*
0–1	2 (1.0)	196	0.1–3.6%	38.4
2–5	8 (4.7)	169	2.0–9.1%	10.0
6–12	16 (5.0)	323	2.9–6.9%	0.32
Total (0–12)	26 (3.9)	668	2.5–5.5%	...
Adults (20–30)	0 (0.0)	148	0.0–2.4%	...

* Incidence data for 1982.^{1, 12}

Table II. Subtyping of carriage and meningitis strains of *H. influenzae* type b from Amsterdam and The Netherlands

Source of strains	Year of isolation	Number tested	Number of strains of																					
			OMP subtype							BT				LPS serotype										
			1*	1a	1b	1c	2	3	others	I	II	IV	V	1	2	3	4	5	6	7	8	9	10	NT
Carriers (Amsterdam)	1987	24	12	3	3	1	2	1	2	19	4	0	1	17	0	0	0	0	0	0	0	3	1	3
Patients (Amsterdam)	1975-1987	97	80	2	0	4	1	5	5	82	12	2	1	81	0	1	0	0	4	3	0	0	5	3
Patients (Netherlands)	1975-1980	80	67	6	2	0	4	1	0	77	3	0	0	74	2	1	1	0	0	0	0	0	0	2
Patients (Netherlands)	1987	96	85	6	1	1	0	0	3	93	3	0	0	92	0	0	0	0	0	0	0	1	0	3

* Subtype 1 is identical with subtype 3L described by Barenkamp *et al.*; ⁵ subtypes 1b with 16L, 2 with 2L and 3 with 6U.

and patients on subtype distribution between carrier and patient strains was analysed by comparing the subtype distribution of strains from carriers 0-5 years old with those from older children. Subtype 1 was found in six of 10 carriers in the younger group and in six of 14 older children, indicating that the common subtype was not more common in the at risk age group (0-5 years).

The LPS serotype distribution of carrier and patient strains was slightly different. LPS-1 was less common among carriers (71%) than among patients (82%) in Amsterdam, but this difference was not significant ($p=0.16$, Fisher's exact test). Ten (42%) of 24 carrier strains and 69 (71%) of 97 patient strains were OMP subtype 1, LPS-1, biotype I indicating that the various subtyping characters were associated. This difference is significant ($\chi^2=5.91$, $p<0.01$; OR=3.45).

Discussion

Subtype OMP 1 of *H. influenzae* type b, commonly isolated from patients with meningitis was found significantly less frequently in carriers when strains isolated in the same city (Amsterdam) were examined. Although the periods of isolation were not the same, this could not explain the difference in subtype distribution because the relative contribution of the common subtype did not vary significantly during the study period in The Netherlands (table I). It is very unlikely that this difference was due to differences in age distribution between carriers and patients, because the relative contribution of subtype 1 strains in carriers below and above 5 years of age was similar (43% vs 60%; the percentage for the whole group was 50%). Unfortunately, it was not possible to draw firm conclusions in this respect because the numbers of strains in each group were too low. Interestingly, we did not find subtype 13L strains among the carrier isolates, as found by Hampton *et al.*,¹⁰ which suggests that carriage strains and disease isolates have a similar restricted geographical distribution.¹¹

Subtypes other than subtype 1 found among carriers were also, rarely, isolated from meningitis patients in this study as described previously.^{5, 8, 11} The 'rare' subtypes are found mostly in patients over 6 years old

with predisposing factors.^{3, 7} This indicates that strains of these uncommon subtypes are less virulent and that only a few subtypes have the pathogenic properties required for invasive disease. It is not yet possible to determine whether OMPs are important for virulence or whether they are just markers for different clones with many more specific characteristics.¹⁸ The combined occurrence of OMP subtype 1 with LPS-1 and biotype I among *H. influenzae* type b isolates from patients (71%) and carriers (42%) supports the clonal hypothesis.

A greater association of LPS-1 with meningitis than with carriage could not be demonstrated, nor was another LPS serotype found exclusively among patients or carriers. A special LPS structure may not be required for invasive disease. In this respect it is interesting to note that Kimura and Hansen have found a reduction in the virulence of *H. influenzae* type b strains following the loss of some LPS epitopes.¹⁹ This result not only indicates that LPS is important for virulence, but also that the capsular polysaccharide alone is insufficient.

The differences in subtype and age distribution of *H. influenzae* type b in carriers and cases indicate that risk of causing disease cannot be deduced directly from data on the carriage of *H. influenzae* type b without taking account of subtype distribution. This conclusion is in agreement with data from Hall and colleagues²⁰ who showed that invasive disease in Alaskan Eskimos was caused by subtypes other than those found among carriers in the same village in the same period. Furthermore, Bijlmer *et al.* did not observe any cases of meningitis in villages in The Gambia where carriage rates rose to 40%.²¹ However, the same subtypes of *H. influenzae* type b were found among cases and carriers in this study and others.^{6, 10} Therefore, carriage would appear to contribute to the spread of *H. influenzae* disease, but in an unpredictable way. The effect of carriage on the acquisition of protective immunity during childhood may be responsible for this unpredictable pattern.

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