EPIDEMIOLOGICAL TYPING

Clonal analysis of *Haemophilus influenzae* type b isolates in the United Kingdom

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Summary. Strains of *Haemophilus influenzae* type b were collected as part of an epidemiological study of bacterial meningitis in the North East Thames Region (NETR) of England. Subclones of *H. influenzae* were identified by outer-membrane protein typing and multilocus enzyme electrophoresis. These were compared with subclones of *H. influenzae* type b isolated from elsewhere in the UK. The subclone ET 12.5/OMP 3L was identified in 68 % of isolates from cases of meningitis from the NETR. In the isolates from elsewhere in the UK, this subclone was identified in 79 % of sterile-site isolates and 91 % of isolates from non-sterile sites. This subclone predominated in patients of different ages and from different ethnic groups, which suggests that non-Caucasians are infected with the subclone predominating locally. Since ET 12.5/OMP 3L subclone is the most abundant strain isolated in virtually all other Western European countries, these results provide additional evidence that the genetic diversity in the *H. influenzae* type b population in Europe is relatively restricted.

Introduction

Haemophilus influenzae type b (Hib) is responsible for 95 % of invasive disease caused by *H. influenzae*. Infections due to Hib are most common in childhood and two-thirds of cases present as meningitis. Before the widespread introduction of Hib vaccination in the UK in October 1992, the incidence of Hib disease was estimated at 26–34 cases/100 000 children under the age of 5 years.^{1,2}

Analysis of outer-membrane protein (OMP) profiles by SDS-PAGE has been used previously to form a Hib subtyping system. This typing scheme distinguishes 21 subtypes of Hib.^{3,4} Further discrimination amongst Hib isolates has been accomplished with multilocus enzyme electrophoresis.⁵ On the basis of variation in electrophoretic mobilities of 17 metabolic enzymes, 182 electrophoretic types (ETs) of Hib have been identified.⁶ This technique has demonstrated that the population structure of Hib is basically clonal.^{7,8} There are striking differences in the clones (determined by ET) and subclones (determined by ET and OMP subtype) predominating in the USA, Western Europe and elsewhere.^{4,9} For example, in Europe, most strains are subclone ET 12.5/OMP 3L whereas in the USA there are several abundant subclones, including ET 1.9/OMP 1H, ET 12.5/OMP 3L and others.^{5,6}

In this study, the OMP subtypes and multilocus enzyme genotypes of Hib isolates collected in 1991 and 1992 as part of an epidemiological survey of bacterial meningitis in the North East Thames Region (NETR) of the UK were determined. The distribution of subclones was compared with that of type b isolates collected from other regions of the UK during the first 3 months of 1991 as part of a survey of the antimicrobial resistance of *H. influenzae*.¹⁰

Materials and methods

Bacterial isolates

NETR isolates. Consultant microbiologists in the NETR identified patients with primary bacterial meningitis and forwarded case details, ethnicity data and isolates to the London Hospital Medical College. During 1991 and 1992, *H. influenzae* caused 121 cases of meningitis, of which 114 (94 %) were type b. Seventy-three (60 %) isolates were available for analysis and these constituted the NETR samples.

Other UK isolates. In a survey of antimicrobial resistance, clinical isolates of all types of *H. influenzae* were collected during the first 3 months of 1991 by 23 laboratories throughout the UK;¹⁰ 41 Hib isolates were identified which had been obtained from the following sites: blood (13), CSF (14), synovial fluid (1), sputum (7), ears (2), eyes (3) and not stated (1).

Figure. SDS-PAGE gel showing Hib OMP subtypes of control strains and seven meningitis isolates. Lanes 1, 6, 14: molecular size markers; 2, 3: controls, OMP subtype 3L; 4, 5: controls, OMP subtype 1H; 7, 8: meningitis isolates of subtype 1H; 9-13 meningitis isolates of subtype 3L.

All strains were confirmed as *H. influenzae* by XV-disk satellitism and a negative result in the porphyrin-production test.¹¹ The capsular type was determined by agglutination with *H. influenzae* type b-specific antiserum (Wellcome).

Statistical analysis

All tests of significance were performed with Epi Info 5.01 (Centers for Disease Control and Prevention, Atlanta, GA 30333, USA). The χ^2 test and Fisher's exact test were used as appropriate for statistical analysis.

OMP subtyping and multilocus enzyme electrophoresis

OMP subtyping was performed by SDS-PAGE of outer-membrane preparations of *H. influenzae* prepared as described by Stull *et al.*¹² Cultures were grown to mid-log phase in brain heart infusion broth supplemented with Fildes's extract 5%. Cells were harvested by centrifugation at 3000 rpm for 20 min and resuspended in 8 ml of 10 mM Tris-acetate buffer, pH 7.2 containing 0.75 M sucrose and 5 mM MgCl₂, to which lysozyme 100 μ g, DNAase 800 μ g and RNAase 800 μ g (all from Sigma) were added. Sonication (4 $\times$ 15 s) was used to disrupt the cells, with cooling on ice between bursts of sonication. The sonicate was centrifuged at 150 000 *g* for 1 h at 4°C and the resulting pellet was

suspended in 1 ml of 10 mM Tris-acetate buffer, pH 7.8, and 1 ml of Triton X-100 4% v/v in the same buffer. After incubation at room temperature for 20 min, centrifugation and resuspension of the pellet was repeated, with a 2% v/v solution of Triton X-100. The detergent-insoluble membrane fractions were heated to 100°C in SDS 1% w/v for 5 min before electrophoresis.

Electrophoresis of the outer-membrane preparations was as described by Barenkamp *et al.*,³ with a modified Laemmli gel system based on the procedure of Lugtenberg *et al.*¹³ Stacking and running gels contained acrylamide 6% and 14% w/v, respectively. OMP subtypes were identified on the basis of the patterns produced by the major protein bands,³ and were confirmed by comparison with Barenkamp reference strains provided by Dr Z. Jordens (Public Health Laboratory, John Radcliffe Hospital, Oxford) (figure). The OMP subtypes of a selection of subtype 1H strains was confirmed by Dr J. Ward (Department of Paediatrics, UCLA Center for Vaccine Research, Harbor-UCLA Medical Center, 1000 West Carson Avenue, Torrance CA 90509, USA).

Electrophoretic types were determined by analysis of allelic variation in 17 chromosomally determined metabolic enzymes.⁵ Electromorphs of each enzyme correspond with alleles on the chromosome. Distinctive combinations represent multilocus genotypes and are designated as electrophoretic types.

Isolates were designated into subclones on the basis of their ET/OMP subtype and clones on the basis of their ET alone, as has been described previously.^{5,6}

Results

NETR isolates

Amongst the 73 isolates from cases of meningitis, the outer-membrane protein subtype 3L was the most common, found in 57 (78%) of cases (table I). The subclone ET 12.5/OMP 3L predominated, accounting for 50 (68%) of the isolates. The next most common subclone from cases of meningitis was ET 1.9/OMP 1H (12%).

Sixty-eight (96%) of 71 isolates where age was

Table I. Multilocus enzyme genotypes (electrophoretic types, ET) and OMP subtypes of *H. influenzae* type b isolates from cases of meningitis in the North East Thames Region

ET	Number (%) of isolates of OMP subtype						Total
	1H	1L	2L	3L	6U	Other	
1.9	9 (12)	0	0	0	0	0	9 (12)
12.5	0	1 (1)	0	50 (68)	0	0	51 (69)
12.8	2 (3)	0	1 (1)	4 (7)	0	0	7 (11)
12.5v*	0	0	0	1 (1)	0	0	1 (1)
12.51†	0	0	0	1 (1)	0	0	1 (1)
12v*	0	0	0	1 (1)	0	1 (1)	1 (2)
25.6	0	0	0	0	2 (3)	0	2 (3)
Total	11 (15)	1 (1)	1 (1)	57 (78)	2 (3)	1 (1)	73 (99)

Percentages do not add up to 100 due to rounding.

*v, variant isolate, each a distinct multilocus genotype.

† Multilocus enzyme genotype similar to, but distinct from, ET 12.5.

Table II. Distribution of subclones of Hib isolates from cases of meningitis in NETR according to age of patient

Age group (years)	Number of isolates of subclone (ET/OMP)				
	12.5/3L	12.8/3L	1.9/1H	Other	Total
< 1	20	2	5	5	32
1-4	25	2	4	5	36
5-19	1	0	0	0	1
20-39	0	0	0	0	0
40-59	1	0	0	0	1
60+	1	0	0	0	1
Total	48	4	9	10	78*

* Details of age unavailable in two cases.

Table III. Distribution of subclones of isolates from cases of Hib meningitis according to ethnicity of patients from NETR

Ethnic group	Number of isolates of subclone (ET/OMP)				
	12.5/3L	12.8/3L	1.9/1H	Other	Total
Caucasian	39	4	7	8	58
Asian	4	0	2	1	7
Black	2	0	0	0	2
Other	2	0	0	0	2
Unknown	2	0	0	2	4
Total	49	4	9	11	73

Table IV. Analysis of subclones of isolates collected from throughout the UK according to culture site

Site	Number of isolates of subclone (ET/OMP)				
	12.5/3L	12.8/3L	12.7/3L	Other	Total
CSF	11	0	1	2*	14
Blood	11	1	1	0	13
Joint	0	0	0	1†	1
Sputum	5	0	1	1‡	7
Ear	2	0	0	0	2
Eye	3	0	0	0	3
Total	32	1	3	4	40

* Subclone 12.8/11L, 12.6/3L.

† Subclone 12.5/14L.

‡ ET unknown, OMP 3L.

Site unknown in one case.

Table V. Analysis of Hib isolates from 23 laboratories in the UK according to subclone and age of patient

Age in years	Number of isolates of subclone (ET/OMP)			
	3L/12.5	3L/12.7	Other	Total
< 5	20	1	3	24
5-19	7	0	1	8
20-29	1	1	0	2
30-39	0	0	0	0
40+	1	1	1	3
Total	29	3	5	37

Details of age unavailable in four cases.

known were from children under the age of 5 years (table II). The subclone ET 12.5/OMP 3L caused 45 cases (63%) of disease in this age group and nine (13%) of the children under 5 years of age were infected with subclone ET 1.9/OMP 1H. The three adult cases were all infected with subclone ET 12.5/OMP 3L.

There were 11 cases in non-Caucasians (table III). Subclone ET 12.5/OMP 3L accounted for eight of these cases. There was no significant difference between the proportion of isolates of this subclone ($p = 1$) or ET 1.9/OMP 1H ($p = 0.6$) in Caucasians and non-Caucasians.

Other UK isolates

Thirty-two (78%) of the isolates belonged to subclone ET 12.5/OMP 3L. Table IV shows a comparison of subclones found from normally sterile sites (CSF, blood, joint) with those from non-sterile sites (sputum, ears, eyes). There were 28 isolates from normally sterile sites and 22 (79%) were the common subclone ET 12.5/OMP 3L. Ten (91%) of 12 isolates from non-sterile sites were of this subclone ($p = 0.2$, comparing the predominant subclone found in normally sterile and non-sterile sites). There were no isolates of subclone ET 1.9/OMP 1H in this collection of isolates.

Table V shows the subclones predominating in

different age groups. Subclone ET 12.5/OMP 3L was isolated from 20 (83%) of the patients < 5 years, and 9 (69%) of the patients > 5 years old.

Discussion

Analysis of the population structure of capsulate *H. influenzae* has shown the species to be clonal, with 182 different ETs found amongst type b isolates. Despite the large number of clones identified, nine clones accounted for 81% of cases of invasive disease worldwide.⁶

Musser *et al.*⁶ identified striking geographical variation in the abundance of clones and subclones. In the USA, 41% of isolates were ET 1.9 and virtually all were of OMP subtype 1H. In Iceland, 82% of isolates were of this electrophoretic type but, in contrast, the majority were of OMP subtype 2L. In Western Europe, 56% of isolates belonged to ET 12.5, and ET 1.9 accounted for a further 14% of cases. Almost all European isolates of ET 1.9 were OMP subtype 1L or 2L.⁶ Others have demonstrated the predominance of subclone ET 12.5/OMP 3L in Western Europe. Van Alphen *et al.*¹¹ identified 75% of isolates from patients in the Netherlands with meningitis to be OMP subtype 1 (Barenkamp OMP subtype 3L). In Wales, Howard *et al.*¹⁵ found 78% of isolates from all invasive Hib disease to be ET 12.5. In contrast, 58 (95%) of 61 isolates of Hib from Pakistani children with lower respiratory tract infection belonged to a subclone rarely seen in the USA or Europe, ET 141/OMP 6U.¹⁶

Although there are geographical differences in the clones and subclones in different continents, there is little evidence to suggest differences in pathogenicity between the clones that cause the majority of invasive Hib disease. In a study from Finland, Hib of OMP

subtype 1c caused proportionally more meningitis and less epiglottitis than strains of OMP subtype 1 (typing nomenclature of van Alphen *et al.*).^{4,17} Barenkamp *et al.*¹⁸ described the OMP subtype 1H as being more transmissible and possibly more virulent than other OMP subtypes within a day-care setting. One particular subclone (ET 21.6/OMP 13L) has been associated with respiratory-tract carriage.^{9,19}

Our data demonstrate that, in the UK, subclone ET 12.5/OMP 3L predominates, as in other parts of Western Europe. There was no difference in the distribution of subclones isolated from Caucasians and non-Caucasians in the NETR study, which suggests that non-Caucasians are infected with the subclone predominating locally.

In the group of isolates from elsewhere in the UK, the subclone predominating in normally sterile body sites (ET 12.5/OMP 3L) was the same as that found in isolates from non-sterile sites. Subclone ET 1.9/OMP 1H appeared only in NETR isolates from cases of meningitis and was not found in the other UK samples. This subclone has not been identified in any other European country but is well documented in the USA.

Overall, the predominant subclone causing meningitis was the same as that causing other forms of Hib disease. This subclone is the most abundant strain of Hib isolated in virtually all other Western European countries, providing additional evidence that the genetic diversity of the Hib population in Europe is relatively restricted.

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