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Subtyping of *Haemophilus influenzae* type b strains from Europe and North America by SDS–PAGE of whole-cell polypeptides: the geographical distribution of subtypes

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SUMMARY

One hundred and nine strains of *Haemophilus influenzae* type b were subtyped by sodium dodecyl sulphate polyacrylamide gel electrophoresis of whole-cell polypeptides. Twenty-one strains from England, 44 from Scotland, 8 from Sweden, 6 from the Netherlands and 30 from the USA were examined. Some of these strains had been subtyped by outer membrane protein analysis; most of the strains had been isolated from cases of invasive disease.

Comparison of polypeptide profiles using the Dice coefficient of similarity showed that the majority of European strains were closely related and formed a single large group. Four smaller groups were identified; three of these included American and European strains, indicating a world-wide distribution of subtypes. However, the common European and American subtypes fell into different groups, indicating the existence of marked geographical variations in subtype frequency.

INTRODUCTION

A number of techniques have been used to subtype strains of *Haemophilus influenzae*. Biotyping (Kilian, 1976) has been widely employed. It discriminates poorly between isolates from invasive disease, most strains falling into biotype 1. Barenkamp, Munson & Granoff (1981*a*) have developed a subtyping method based on the analysis of outer membrane proteins (OMP) by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). More than 20 OMP subtypes have been isolated from cases of invasive disease in the USA, although five subtypes predominate (Barenkamp, Munson & Granoff, 1981*b*). Van Alphen and colleagues (van Alphen *et al.* 1983, 1987) have developed a similar OMP subtyping system and have shown that a single subtype predominates in Europe. A multilocus genetic typing system based on the electrophoresis of chromosomally encoded metabolic enzymes has also been developed (Musser *et al.* 1985*a*,*b*; Porras *et al.* 1986) to provide a powerful molecular epidemiological approach. This has shown that *H influenzae* has a clonal population structure and that there is limited genetic diversity among strains isolated from cases of invasive disease.

Our own studies (Paterson, Macsween & Pennington, 1987) have shown that SDS–PAGE of whole-cell polypeptides can be used to subtype strains of *H*

influenzae. In our preliminary study strains from north-east Scotland were examined; in the present paper we report the results of subtyping by SDS–PAGE of whole-cell proteins of type b strains of *H. influenzae* isolated in England in Scotland. These strains have also been compared with isolates from Sweden, the Netherlands and the USA.

MATERIALS AND METHODS

Bacterial strains

A total of 109 strains of capsulated *Haemophilus influenzae* type b was examined in this study. Twenty-one English strains isolated in 1986 and 1987, and selected to give as wide a geographical distribution as possible, were provided by Dr M. P. E. Slack, the Nuffield Department of Pathology, John Radcliffe Hospital, Oxford. Twenty-three strains isolated in the USA between 1982 and 1985 and seven OMP subtype reference strains were provided by the Edward Mallinckrodt Department of Pediatrics, Washington University School of Medicine, Saint Louis, USA, and eight strains were obtained from the C.C.U.G. Culture Collection, Department of Clinical Bacteriology, University of Göteborg, Sweden. Six OMP reference strains were supplied by Dr L. van Alphen, Vakgroep Medische Microbiologie, University of Amsterdam, the Netherlands, and 44 strains were obtained from our own diagnostic laboratories in the Department of Medical Microbiology, University of Aberdeen. The strains were isolated beween 1975 and 1987 and most were cultured from blood or cerebrospinal fluid from patients with meningitis (44% of samples), epiglottitis (13·8% of samples) or other invasive diseases. A few strains had been cultured from nasal and throat swabs from carriers.

On receipt all strains were sub-cultured on chocolate agar (Oxoid DST Agar, 7% heated blood) and stored as freeze-dried ampoules.

Preparation of bacteria for whole-cell analysis

The strains were cultured overnight on chocolate agar at 37 °C in the presence of CO_2 (5%). Single colonies were sub-cultured on chocolate agar and harvested in 10 mM-Tris-HCl, pH 6·8, 1 mM-EDTA using a sterile bent Pasteur pipette. After centrifugation at 7500 g for 8 min in a Beckman Microfuge B the supernatant was discarded, the pellet washed in sterile double-distilled water (DDW) and re-centrifuged. The resulting pellet was suspended in an equal volume of DDW and the cells disrupted in wet ice for 2 min with an ultrasound probe emitting 45 W at maximum power. The suspension was stored at −70 °C till required.

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE gel electrophoresis was performed using the method of Laemmli (1970) as modified by Krikler, Pennington & Petrie (1986). 11% polyacrylamide resolving gels overlaid with 3·6% stacking gels were cast using a BioRad Protean II water-cooled system. Whole-cell sonicates were prepared for SDS-PAGE by solubilizing in sample buffer (SDS 2%, w/v, 2-mercaptoethanol 5%, w/v, glycerol 10%, v/v, 0·05 mM-Tris-HCl, pH 6·8) and heating in a boiling water bath for 5 min. Immediately before being loaded into the wells the samples were microfuged

briefly to remove any gross cell debris. Gels were run at a constant 30 mA till the bromophenol blue marker had just migrated beyond the bottom of the gel. Polypeptides were stained in 0·25% Coomassie brilliant blue in 45·5% methanol, 9% acetic acid; destained in 50% methanol, 3·5% acetic acid and fixed in 7% acetic acid. Pharmacia low molecular weight standards used throughout were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20·1 kDa) and lactalbumin (14·4 kDa). Polypeptide profiles were compared using the Dice coefficient of similarity (Dice, 1945), where the percentage similarity between two strains (%S) =

$$\frac{\text{number of matching bands} \times 2}{\text{total number of bands in both strains}} \times 100.$$

Comparisons were performed on wet gels using a light box to obtain maximum definition. Polypeptide similarity matrices were then constructed (Paterson, Macsween & Pennington, 1987). Gel analyses giving the Dice coefficient were performed for strains from each country, strains from each country, strains from the Netherlands and Sweden being treated as one group because of the small sample size. Representative strains from each national group and sub-group were then compared. Similarities of 95% and greater were considered to define groups.

RESULTS

Comparison of polypeptide profiles

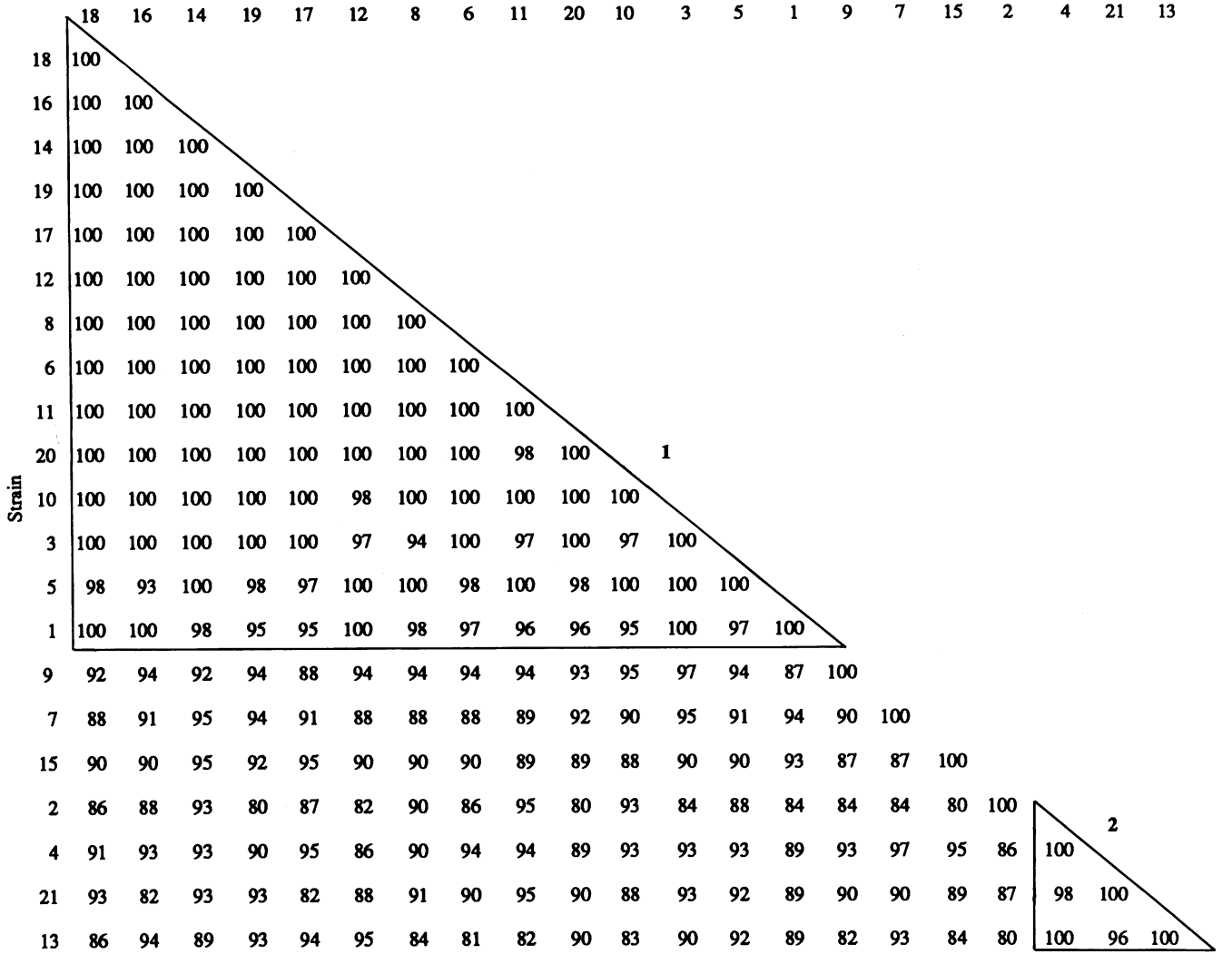
Every strain of *H. influenzae* type b in the collection was electrophoresed several times. Repeat analyses showed that the polypeptide band patterns were highly reproducible. Alterations in the methods of sample preparation did not affect the band patterns. For example (results not shown), strains were grown on chocolate agar and in brain heart infusion supplemented with NAD and haemin; strains were harvested in DDW, phosphate buffered saline, 10 mM-Tris–HCl (pH 6·8) 1 mM-EDTA or digestion buffer (50 mM-Tris–HCl, pH 7·5, 10 mM-$MgCl_2$, DNAse 5 μg/ml). In no instance was there any discernible difference in the polypeptide band patterns on the gels.

No strain gave fewer than 39 polypeptide bands suitable for analysis with 48 bands being the average. The lowest coefficient of similarity calculated was 74%; in the majority of comparisons coefficients of similarity were greater than 90%.

Comparison of English and Scottish strains

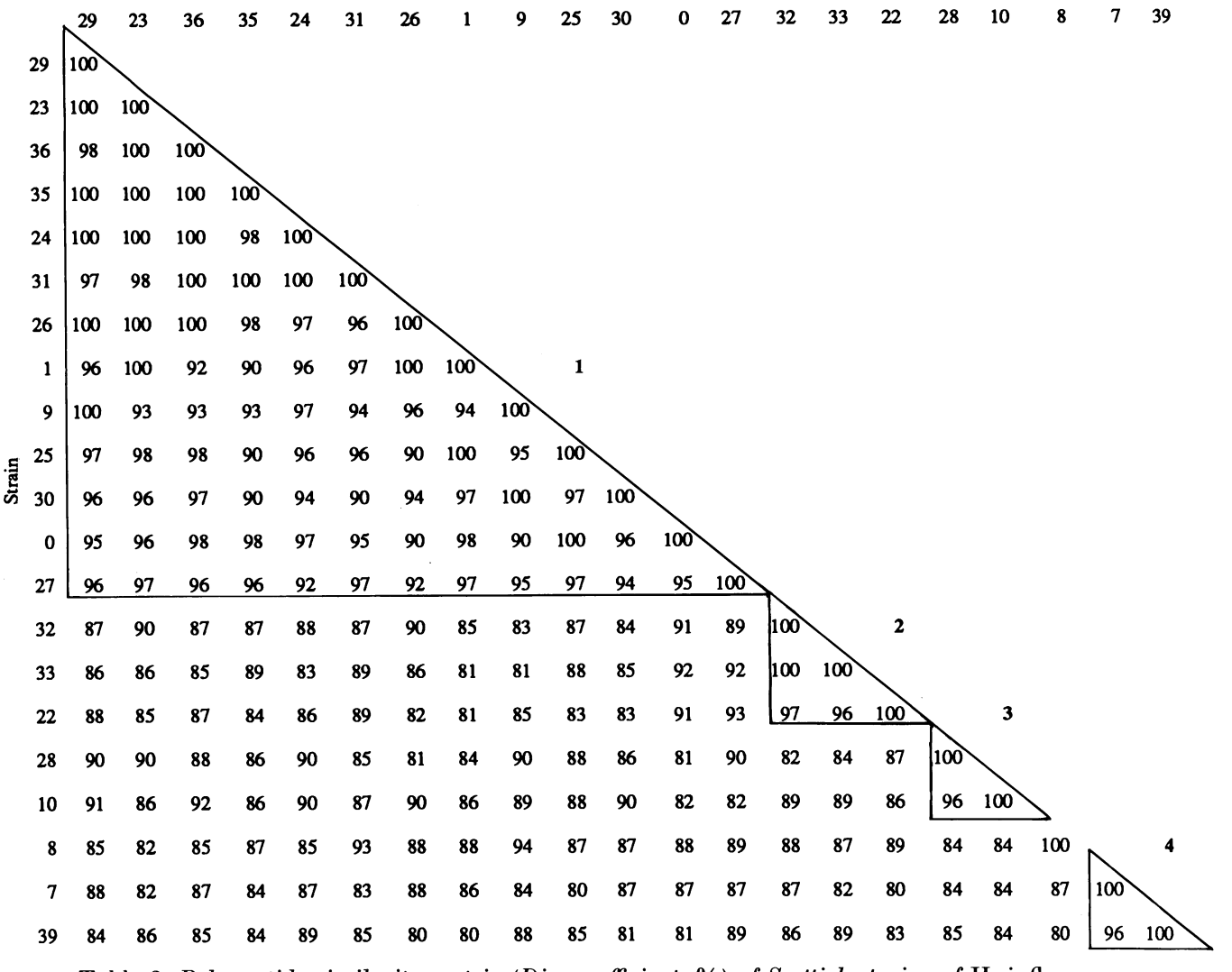
Table 1 show a polypeptide similarity matrix of the English strains. Two thirds of isolates (strains 18-1) formed a homogeneous group, most of the strains having identical polypeptide profiles. Strains 9, 7, 15 and 2 were less closely related, and strains 4, 21 and 13 formed another group (Group 2) being closely related to each other but not to the remaining strains.

An analysis of all Aberdeen isolates, including the strains analysed by Paterson, Macsween & Pennington (1987), had shown that strains fell into at least four groups. These are shown in the matrix illustrated in Table 2, which is derived from



Strain	18	16	14	19	17	12	8	6	11	20	10	3	5	1	9	7	15	2	4	21	13
18	100																				
16	100	100																			
14	100	100	100																		
19	100	100	100	100																	
17	100	100	100	100	100																
12	100	100	100	100	100	100															
8	100	100	100	100	100	100	100														
6	100	100	100	100	100	100	100	100													
11	100	100	100	100	100	100	100	100	100												
20	100	100	100	100	100	100	100	100	98	100		1									
10	100	100	100	100	100	98	100	100	100	100	100										
3	100	100	100	100	100	97	94	100	97	100	97	100									
5	98	93	100	98	97	100	100	98	100	98	100	100	100								
1	100	100	98	95	95	100	98	97	96	96	95	100	97	100							
9	92	94	92	94	88	94	94	94	94	93	95	97	94	87	100						
7	88	91	95	94	91	88	88	88	89	92	90	95	91	94	90	100					
15	90	90	95	92	95	90	90	90	89	89	88	90	90	93	87	87	100				
2	86	88	93	80	87	82	90	86	95	80	93	84	88	84	84	84	80	100			
4	91	93	93	90	95	86	90	94	94	89	93	93	93	89	93	97	95	86	100		
21	93	82	93	93	82	88	91	90	95	90	88	93	92	89	90	90	89	87	98	100	
13	86	94	89	93	94	95	84	81	82	90	83	90	92	89	82	93	84	80	100	96	100

Table 1. *Polypeptide similarity matrix* (*Dice coefficient*, %) *of English strains of* H. influenzae
(**1, 2**: groups of strains with closely related polypeptide profiles.)



Strain	29	23	36	35	24	31	26	1	9	25	30	0	27	32	33	22	28	10	8	7	39
29	100																				
23	100	100																			
36	98	100	100																		
35	100	100	100	100																	
24	100	100	100	98	100																
31	97	98	100	100	100	100															
26	100	100	100	98	97	96	100														
1	96	100	92	90	96	97	100	100		1											
9	100	93	93	93	97	94	96	94	100												
25	97	98	98	90	96	96	90	100	95	100											
30	96	96	97	90	94	90	94	97	100	97	100										
0	95	96	98	98	97	95	90	98	90	100	96	100									
27	96	97	96	96	92	97	92	97	95	97	94	95	100								
32	87	90	87	87	88	87	90	85	83	87	84	91	89	100		2					
33	86	86	85	89	83	89	86	81	81	88	85	92	92	100	100						
22	88	85	87	84	86	89	82	81	85	83	83	91	93	97	96	100		3			
28	90	90	88	86	90	85	81	84	90	88	86	81	90	82	84	87	100				
10	91	86	92	86	90	87	90	86	89	88	90	82	82	89	89	86	96	100			
8	85	82	85	87	85	93	88	88	94	87	87	88	89	88	87	89	84	84	100		4
7	88	82	87	84	87	83	88	86	84	80	87	87	87	87	82	80	84	84	87	100	
39	84	86	85	84	89	85	80	80	88	85	81	81	89	86	89	83	85	84	80	96	100

Table 2. *Polypeptide similarity matrix (Dice coefficient, %) of Scottish strains of* H. influenzae
(**1–4**: groups of strains with closely related polypeptide profiles.)

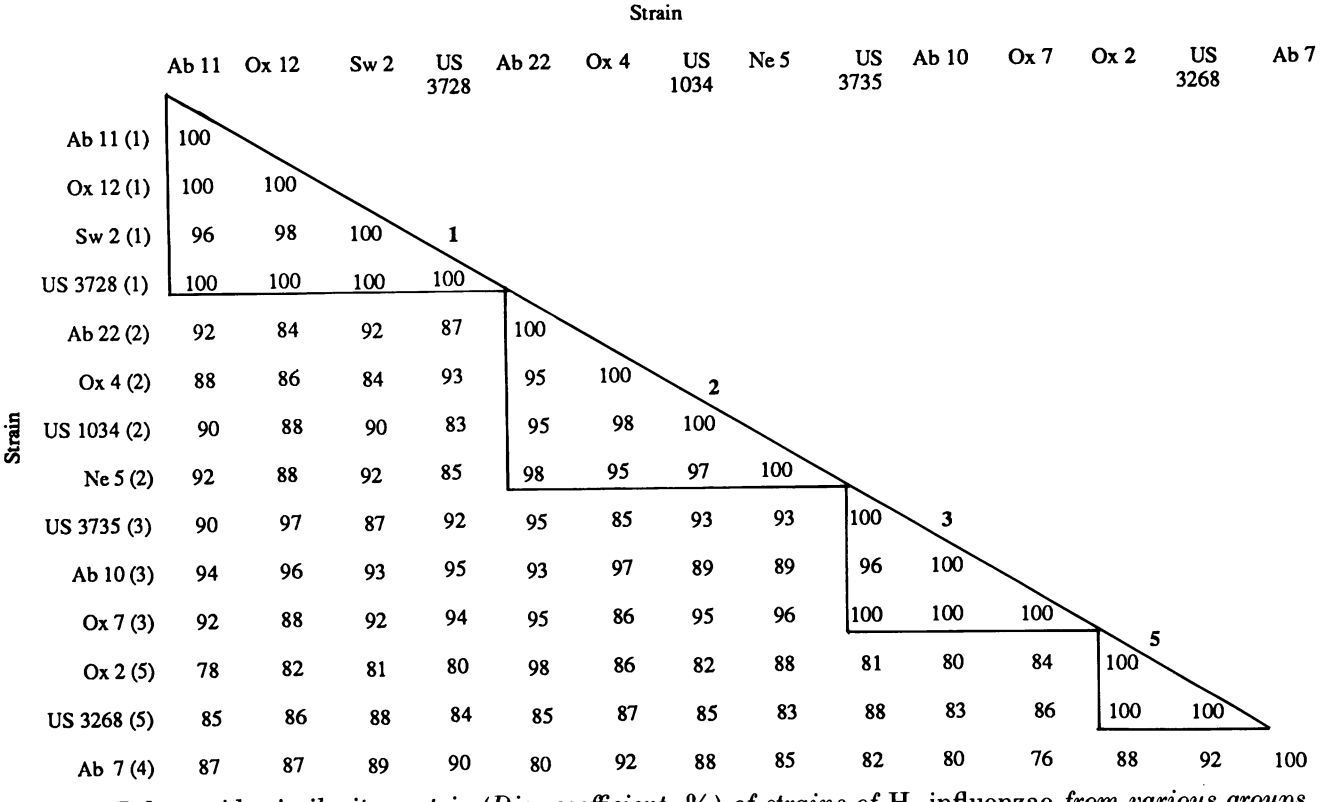


Table 3. *Polypeptide similarity matrix (Dice coefficient, %) of strains of* H. influenzae *from various groups*

(Ab: Scottish strains; Ox: English strains; Sw: Swedish strains; Ne: Netherlands strains US: USA strains. **1, 2, 3, 5**: groups of strains with closely related polypeptide profiles.)

a gel analysing a subset of strains belonging to these groups. As with the English strains most Scottish strains fell into a single group. This contained 36 of the 44 strains analysed. The strains forming this large group and the three small groups (strains 32, 33 and 22 (group 2), strains 10 and 28 (group 3) and strains 7 and 39 (group 4)) were compared with English strains. Typical results are shown in Table 3. The strains in the two large groups were closely related and formed a single group, which was termed group 1. The strains in the English and Scottish group 2 categories were also closely related and formed a single group (2). The Scottish strains 10 and 28 were closely related to English strain 7 and formed a third group (3).

Comparison of UK strains with isolates from Sweden, the Netherlands and the USA

The UK strains were compared with a small collection of Swedish strains and strains from the Netherlands representative of Van Alphen's (van Alphen *et al.* 1983) outer membrane protein subtypes 1, 1a, 1b, 2 and 3. Typical results are shown in Table 3, which also shows comparisons with strains from the USA. All the Swedish strains fell into our group 1, as did the OMP subtype 1, 1a and 1b strains from the Netherlands. The OMP subtype 3 strain was not closely related to any UK strain but the OMP subtype 2 (Ne 5) strain was closely related to the UK strains in group 2.

Comparisons of UK strains with isolates from the USA which had been OMP subtyped by Barenkamp's method (Barenkamp, Munson & Granoff, 1981*a*) showed that a close relationship existed between the strains in group 2 and a strain with OMP subtype 15L, and that the strains in group 3 were closely related to a group of OMP unsubtyped strains and one with OMP subtype 1 H. However, only one of the 30 American strains tested showed a close relationship to the European group 1 strains, this isolate (OMP subtype not determined) showing a polypeptide profile which was indistinguishable from that of group 1 strains isolated in Scotland, England and Sweden. One UK strain (Ox 2) was closely related to a strain from the USA (3268); neither of these strains were closely related to the other strains in their national collections.

DISCUSSION

Our previous work (Paterson, Macsween & Pennington, 1987) has shown that SDS–PAGE of whole-cell polypeptides can be used to sub-type strains of *H. influenzae*, and that the majority of type b strains isolated in N.E. Scotland between 1983 and 1986 were closely related. In the present study, examination of additional isolates from N.E. Scotland and a collection of strains of diverse geographical origins within England has shown that a similar population structure exists in the UK as a whole, the majority of type b isolates being closely related. Studies of European strains of *H. influenzae* type b by enzyme electrophoresis (Porras *et al.* 1986) and by outer membrane protein subtyping and lipo-polysaccharide serotyping (van Alphen *et al.* 1987) have given similar results, showing that a single subtype predominates in Finland, Denmark, Norway, Sweden, Germany, France, the UK, and the Netherlands. It has been suggested by Van Alphen and colleagues (van Alphen *et al.* 1987) that the strains which form

this common European subtype are all descendants from one ancestral clone. The close relationship between the SDS–PAGE whole-cell polypeptide profiles of group 1 UK strains to strains from the Netherlands belonging to the common European subtype (van Alphen OMP subtype 1) and to all the Swedish strains tested, indicates that all these strains are clonally related, and that strains belonging to the major UK group are members of the common European subtype.

It has been observed previously that clonal cell lines of *H. influenzae* type b have a world-wide distribution, a conspicuous example being the isolation of strains of Barenkamp OMP subtype 3L/electropherotype 12/biotype II from Australia, the Netherlands and throughout the US. The finding in the present study that European strains in groups 1, 2 and 4 were very closely related to American isolates provides further evidence of the wide geographical distribution of subtypes. However, the frequency of subtypes varies markedly from one locality to another, a particularly striking example being the virtual absence of the European common subtype from Iceland and the predominance there of strains of van Alphen OMP subtype 2, lipopolysaccharide serotype 9 and biotype 1 (van Alphen *et al*, 1987). Another striking difference is shown by the demonstration in this study that strains with SDS–PAGE whole-cell polypeptide profiles closely related to strains of Barenkamp OMP subtype 1 H are rare in Europe (3 of 79 strains examined); in the United States this is the most frequent outer membrane subtype isolated from cases of invasive disease (Hampton, Barenkamp & Granoff, 1983). The absence of strains of this OMP subtype from a collection of isolates made in the USA between 1934 and 1954 and their abundance after 1977 has led Barenkamp and colleagues (Barenkamp, Granoff & Pittman, 1983) to suggest that the frequency of this subtype increased dramatically in the USA between these periods. As similar strains occur in Europe but are still rare there it would seem prudent to monitor possible changes in their frequency – changes which might affct the incidence of invasive disease – in Europe.

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