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Haemophilus influenzae subtyping by SDS–PAGE of whole-cell polypeptides

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SUMMARY

Strains of *Haemophilus influenzae* isolated from patients in N.E. Scotland between 1983 and 1986 have been subtyped by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of whole-cell polypeptides. Gels were stained with Coomassie blue and polypeptide profiles were analysed using the Dice coefficient of similarity.

Type b strains were all closely related, the 19 strains analysed being grouped at a 90% similarity level into one large (13 strains) and one small (3 strains) cluster with 3 strains being ungrouped. Thirty-six non-typable, epidemiologically unrelated strains were subtyped; one pair of strains had indistinguishable polypeptide profiles. The polypeptide profiles of the remaining strains showed much heterogeneity, although groups of strains isolated from the same patient over short periods showed indistinguishable profiles.

INTRODUCTION

Haemophilus influenzae strains can be subtyped using the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) outer membrane protein (OMP) profile. Studies on serotype b strains collected in the USA have shown that a considerable number of OMP subtypes are associated with disease (Barenkamp, Munson & Granoff, 1981), although one subtype, 1H, accounts for a significant number of the strains examined. OMP subtyping has also been used to provide evidence of nosocomial transmission (Barton, Granoff & Barenkamp, 1983) and the spread of infection in day-care centres (Barenkamp, Granoff & Munson, 1981). OMP studies in Europe have shown, however, that Swedish (Porras *et al.* 1986*b*) and Dutch (van Alphen, 1983) strains show much less variation than American strains. OMP subtyping represents a considerable advance in typing technology, being superior to the biotyping scheme described by Kilian (1976) which fails to distinguish between the majority of serotype b strains.

Recent studies using a multilocus genetic typing system based on the electrophoresis of chromosomally encoded enzymes (Musser *et al.* 1985, 1986; Porras *et al.* 1986*a*, *b*) have shown, however, that like capsular typing and biotyping, OMP profiles do not fully reflect strains differences. Using the techniques of population genetics, these analyses of enzyme electrophoretic type (ET) have shown that *H. influenzae* is extremely variable, and have produced evidence supporting the hypothesis that this organism has a clonal population structure. The demon-

stration that *H. influenzae* strains show much genetic diversity stimulated us to carry out a study of strains isolated in the Grampian region, north-east Scotland. SDS-PAGE of whole cell polypeptides was chosen as an analytical method. This technique was chosen in preference to OMP profile analysis because it was expected to be more discriminatory in view of the much smaller number of markers examined in OMP typing and because of the requirement that clonal analysis has for a large number of diverse markers (Diamond, 1986). The large number of markers that can be simultaneously generated in a single electrophoretic run also give whole cell polypeptide analysis a significant technical advantage over classical allozyme electrophoresis.

MATERIALS AND METHODS

Bacterial strains

Capsulate *H. influenzae* strains were obtained from our own diagnostic laboratory. They had been isolated between 1983 and 1986 by blood culture or cerebrospinal fluid culture from patients with invasive disease. Non-capsulate strains were obtained from samples submitted to our laboratory during 1985. The isolates were mainly from the respiratory tract of adults and children.

Polypeptide analysis – preparation of material

The organisms were cultured on 'chocolate' agar plates incubated at 37 °C for 24 h. Colonies were inoculated into 100 ml of tryptose phosphate broth supplemented with Fildes extract and incubated for 24 h in air in an orbital incubator at 37 °C.

The broth culture was centrifuged at 2500 **g** for 15 min and the supernatant discarded. The cell pellet was placed in a 1·5 ml microfuge tube and centrifuged for 5 min. The pellet produced was washed in an equal volume of distilled water and disrupted for 3 min with an ultrasound probe emitting 45 W at maximum power.

Polyacrylamide gel electrophoresis (PAGE)

A modified version of the SDS-PAGE technique of Laemmli (1970) was used (Krikler, Pennington & Petrie, 1986). Specimens were prepared for PAGE by boiling for 5 min in sodium dodecyl sulphate (SDS) 2% w/v, 2-mercaptoethanol 5% v/v, glycerol 10% v/v and 0·05 M Tris-HCl, pH 6·8. They were applied to wells in a 3·6% acrylamide stacking gel over a separating slab gel of 10% acrylamide. Gels were run using the buffers described by Laemmli and until the bromophenol blue marker had reached the bottom of the gel. Proteins were stained with Coomassie blue.

Polypeptide profile comparisons were made using the Dice coefficient of similarity (Dice, 1945) where the average percentage similarity between two strains

$$(\%S) = \frac{\text{number of matching bands} \times 2}{\text{total number of bands in both strains}} \times 100.$$

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Outer membrane protein (OMP) analysis

Sodium *N*-lauryl sarcosinate insoluble proteins (OMP) were prepared by the rapid (ROMP) method of Carlone *et al.* (1986).

Biotyping

The biotype was determined by the method of Mehtar & Afshar (1983), which tested strains for the production of urease, indole, and ornithine decarboxylase.

RESULTS

Type b strains, origin and biotype

Nineteen strains of *H. influenzae* type b from systemic infections were studied. Table 1 shows details of the source of the strains and their biotypes. Meningitis (8 isolates) predominated as the commonest clinical diagnosis although 7 strains were isolated from cases diagnosed definitively or presumptively as epiglottitis. The addresses of patients were evenly divided between Aberdeen (10) and small towns in the largely rural hinterland (8), reflecting the approximately equal division of population between Aberdeen and the rest of the Grampian region served by the laboratory. One organism was isolated from a child with meningitis whose illness presented *in transit* between Kent and Aberdeen. With one exception (biotype II) strains were biotype I.

Subtyping by SDS–PAGE analysis of whole cell polypeptides

Preliminary gel analyses showed all type b strains to be closely related. Repeat analyses were done in triplicate and showed that differences in band pattern were highly reproducible. Strains were analysed in detail in single slab gel runs, and Fig. 1 (*a*) shows the result of such a run stained with Coomassie blue. Table 2 shows the polypeptide similarity matrix constructed from this analysis using the Dice coefficient. Strains could readily be grouped. A large cluster defined by a 90% or greater coefficient of similarity contained 13 strains; three of the remaining strains were similarly but separately grouped, with the remaining three strains being distinguishable from each other and from the other two clusters. Both clusters contained strains isolated from patients with meningitis and epiglottitis and strains isolated at widely differing times during the study period. Three strains with identical polypeptide profiles formed a sub-group in the large cluster, and, although all were isolated from patients living in Aberdeen, the isolations were made in different years and from patients with different manifestations of infection. A similar subgroup was identified in the small cluster where two strains differed only in a single polypeptide. Again both were isolated from patients living in Aberdeen but were associated with different manifestations of infection and were isolated in different years.

Outer membrane proteins of type b strains

Preparation of outer membrane proteins by the ROMP procedure showed that the major outer membrane protein (OMP) of all strains in the two clusters defined

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Table 1. *Source and biotype of* Haemophilus influenzae *type b strains.*

Strain number	Year of isolation	Clinical diagnosis	Site of isolation	Address of patient	Biotype
14	1983	Meningitis	CSF	Aberdeen	I
12	1983	Meningitis	CSF	Portsoy	I
15	1983	Meningitis	CSF	Lhanbryde	I
18	1984	Meningitis	CSF	Aberdeen	I
2	1985	Meningitis	CSF	Macduff	I
5	1986	Meningitis	CSF	Aberdeen	I
4	1986	Meningitis	CSF	Ardoe	I
9	1986	Meningitis	Blood	Chatham, Kent	I
17	1984	Epiglottitis	Blood	Aberdeen	II
10	1985	Epiglottitis	Blood	Aberdeen	I
3	1985	Epiglottitis	Blood and Throat	Oldmeldrum	I
7	1986	Epiglottitis	Blood	Aberdeen	I
6	1986	Epiglottitis	Blood	Fraserburgh	I
19	1986	Epiglottitis	Blood	Skene	I
16	1984	Epiglottitis	Blood	Aberdeen	I
13	1983	Arthritis	Blood	Aberdeen	I
1	1985	Pneumonia	Blood	Kemnay	I
8	1986	Septicaemia	Blood	Aberdeen	I
11	1986	Septicaemia	Blood	Rosehearty	I

by whole cell polypeptide analysis had an apparent mol. wt. of 45000 (Fig. 2). Of the remaining isolates, strains 11 and 17 lacked this polypeptide and the major OMP had an apparent mol. wt. of 43000. The major OMP of strain 18 was identical and that of the strains in the two clusters.

Antibiotic sensitivity of type b strains

β-lactamase production was not detected in any strains. Strain 13 was unique in the collection in being chloramphenicol resistant.

Subtyping of non-typable strains

Thirty-six strains were subtyped by SDS–PAGE of whole cell polypeptides. No large clusters of strains could be identified. Fig. 1(*b*) shows typical results of an SDS–PAGE whole cell polypeptide analysis and the polypeptide similarity matrix of the strains analysed in this gel is shown in Table 3. Strain 10 (isolated from the sputum of an adult) and strain 12 (isolated from upper respiratory tract of an infant) had identical polypeptide profiles. The degree of relationship of the polypeptide profiles of the remaining strains lay between 87 and 26%. None of the non-typable strains (strains 2–17) was closely related to the type b strain included in the analysis (strain 1); the degree of relationship was between 29 and 63%.

Non-typable strains obtained on different occasions up to 9 days apart from individual patients were also analysed; 27 isolates from 11 patients were examined. In every instance all isolates from a single patient yielded indistinguishable whole cell polypeptide profiles. The results of an SDS–PAGE analysis of 9 strains from 4 patients are shown in Fig. 4.

Epidemiologically related strains from different individuals also showed indistinguishable polypeptide profiles; a non-typable isolate from a high vaginal

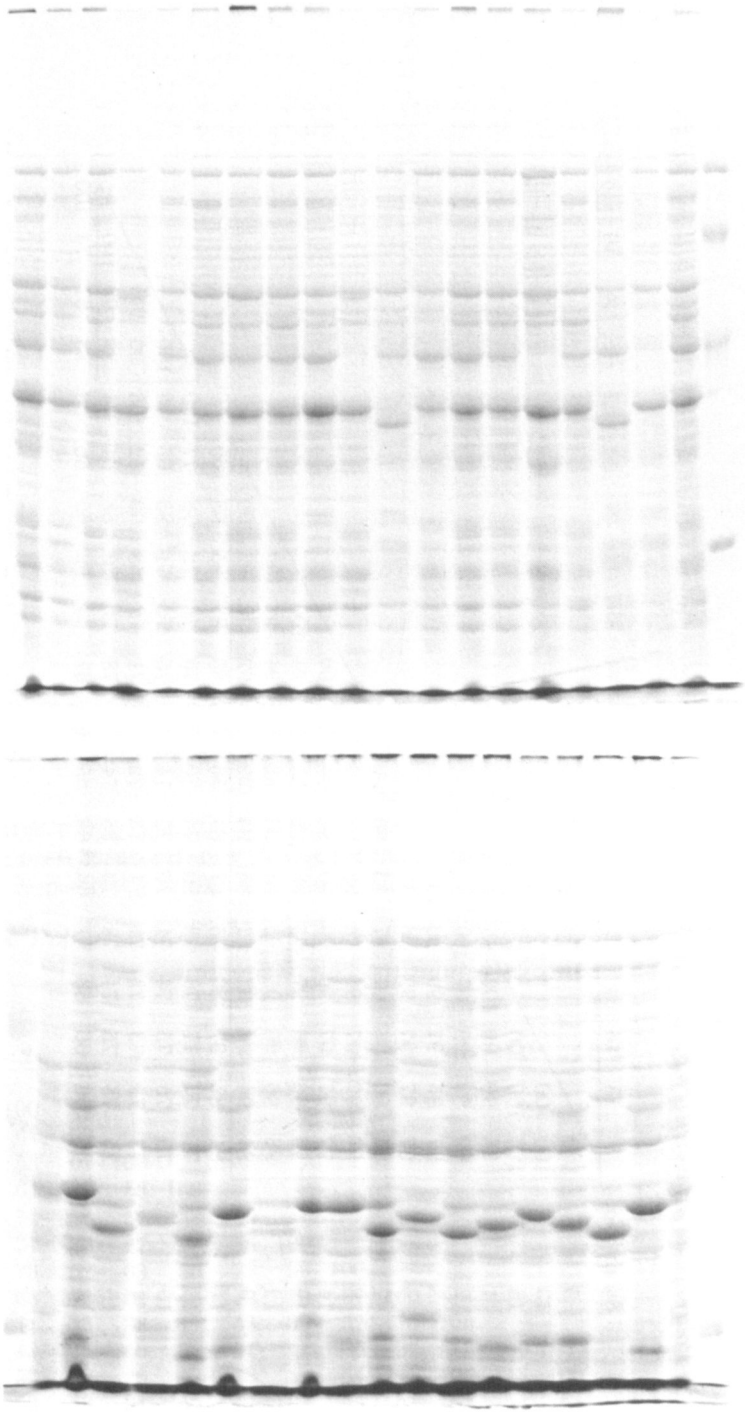

Fig. 1. (*a*) SDS–PAGE of 18 *H. influenzae* type b strains. Tracks 1–18: strains 1–18; track 19: strain 1; track 20: mol. wt. markers. (*b*) SDS–PAGE of 16 *H. influenzae* non-typable strains (tracks 3–18) and 1 type b strain (tracks 2 and 19). Tracks 3, 5, 6, 10, 12, 13, 14, 15: strains from upper respiratory tract of infants; tracks 4, 7, 8, 9, 11, 16, 17, 18: strains from adult sputum; tracks 1 and 20: mol. wt. markers.

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Table 2. *Polypeptide similarity matrix* (*Dice coefficient*, %) *of* Haemophilus influenzae *type b strains described in Table 1.*

Strain No.	Strains 5	13	16	1	2	3	6	7	8	14	19	12	9	11	17	15	4	10	18
5	100																		
13	100	100																	
16	100	100	100																
1	96	99	99	100															
2	99	99	99	97	100														
3	97	95	97	99	96	100													
6	97	97	97	99	96	97	100												
7	94	96	96	97	97	96	96	100											
8	97	97	97	96	96	98	97	94	100										
14	97	97	97	96	96	95	95	94	98	100									
19	96	96	96	97	95	96	96	95	94	94	100								
12	96	96	96	95	95	94	93	92	96	94	92	100							
9	94	94	94	92	97	86	94	92	94	92	92	92	100						
11	84	87	87	86	85	87	84	86	90	85	83	85	88	100					
17	77	77	77	76	78	80	74	70	77	75	78	72	78	77	100				
15	75	75	75	74	74	76	75	74	76	75	72	71	85	68	65	100			
4	73	73	73	72	71	71	73	72	71	71	72	68	69	62	65	90	100		
10	75	75	75	74	73	73	75	74	73	73	76	70	68	64	64	89	99	100	
18	66	66	66	63	67	64	66	65	64	67	63	62	65	66	58	83	67	74	100

Groups of strains with closely related polypeptide profiles

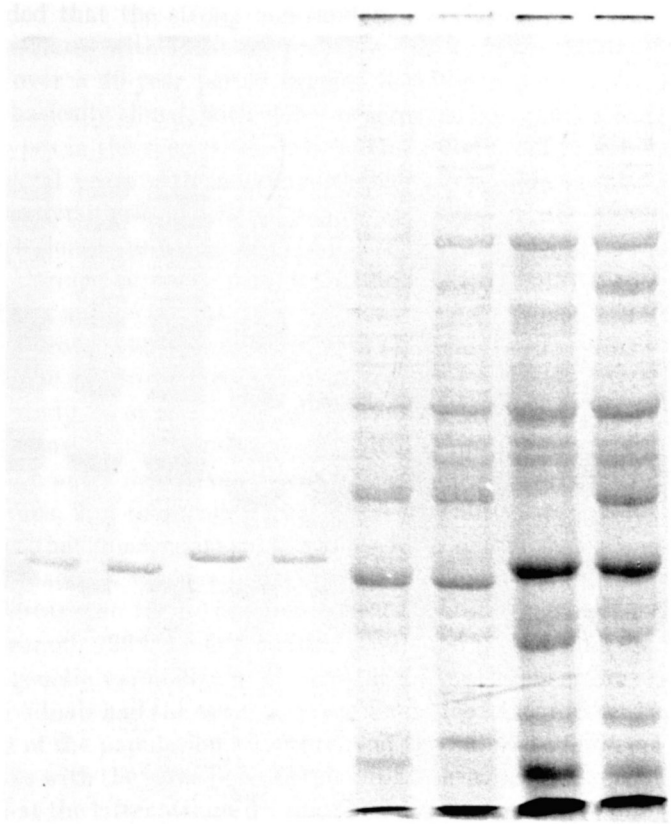

Fig. 2. SDS–PAGE of outer membrane proteins and whole cell proteins of type b *Haemophilus influenzae* strains. Tracks 1–4: outer membrane proteins of strains 17, 11, 15 and 1 respectively; tracks 5–8: whole-cell proteins of same strains.

Table 3. *Polypeptide similarity matrix* (*Dice coefficient* %) *of* Haemophilus influenzae *non-capsulated strains*

	Strains																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	100																
2	45	100															
3	54	64	100														
4	34	53	67	100													
5	42	64	48	56	100												
6	48	74	54	57	38	100											
7	29	31	36	27	29	58	100										
8	40	73	52	63	55	73	39	100									
9	44	63	48	52	45	48	49	87	100								
10	36	41	33	38	47	46	33	67	70	100							
11	39	43	35	40	38	51	41	55	37	45	100						
12	31	52	38	26	42	28	40	64	45	100	48	100					
13	40	53	49	34	55	44	37	54	51	62	44	64	100				
14	55	52	52	51	51	49	43	48	48	54	43	56	67	100			
15	47	58	68	53	59	43	40	58	51	62	66	69	80	67	100		
16	47	43	58	63	43	41	41	61	40	55	46	67	59	61	71	100	
17	63	60	65	63	48	52	37	58	54	51	44	53	66	65	71	63	100

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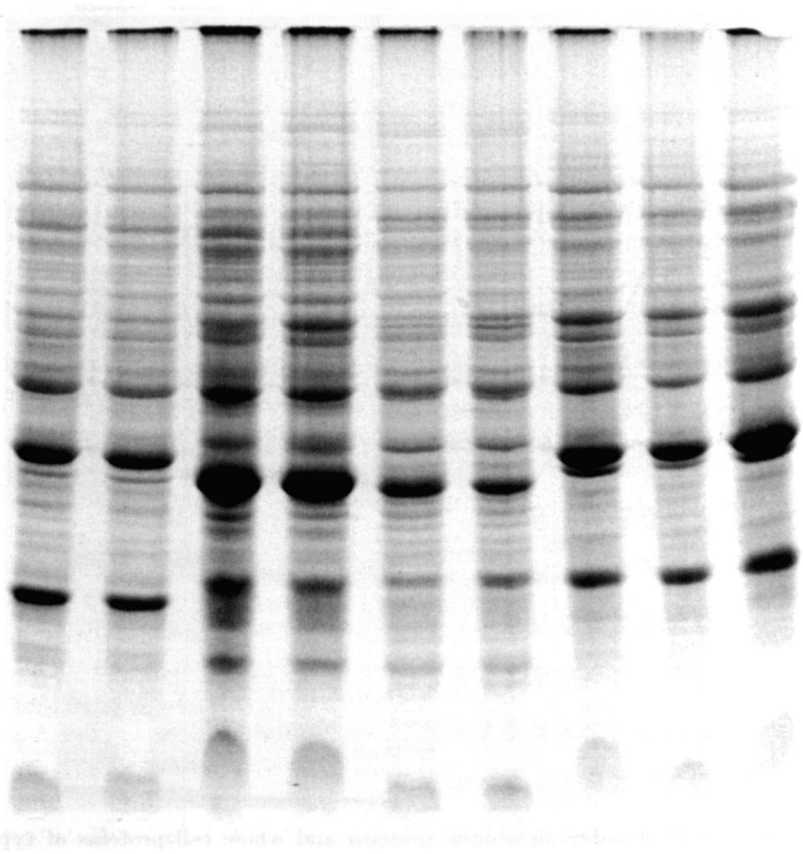

Fig. 3. SDS–PAGE of 9 *Haemophilus influenzae* non-typable strains from 4 patients. Tracks 1, 2: isolates from 2 sputum samples taken independently on same day from patient A; tracks 3, 4: isolates from sputum samples taken 2 days apart from patient B; tracks 5, 6: isolates from nasal catheter tip and eye swab taken 1 day apart from patient C; tracks 7, 8 and 9: isolates from sputum samples taken independently on same day and on succeeding day from patient D.

swab from a patient with prolonged rupture of membranes at 32 weeks of pregnancy was identical in this respect with an isolate from a throat swab from the infant born subsequently.

DISCUSSION

Our results clearly show that capsulate and noncapsulate isolates of *H. influenzae* from patients in N.E. Scotland demonstrate much variability. While our results cannot be directly compared with those of others, due to the use of different subtyping techniques, the degree and kind of variability shown in our study closely resembles that demonstrated by recent studies on American and Swedish strains which have used the classical population genetic technique of multilocus enzyme gel electrophoresis (Musser *et al.* 1985, 1986; Porras *et al.* 1986*a*, *b*). These workers

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have concluded that the strong non-random association of characters and the recovery of isolates with identical properties in widely separated geographical regions and over a 40-year period suggest that the population structure of *H. influenzae* is basically clonal, with clones of serotype b forming a restricted subset of the genotypes in the species as a whole. Our isolation of type b strains over a period of several years with indistinguishable polypeptide profiles lends strong support to the hypothesis of a clonal population structure, and also indicates that more than one clone expressing the type b capsular polysaccharide was circulating in Aberdeen during the study period. Our finding that, with the exception of a small number of unique strains, type b strains from Grampian fall into one large and one small group, can be compared with the analysis of strains from Gothenburg by enzyme electropherotype conducted by Porras *et al.* (1986*b*). In the Gothenburg study 26 of 29 strains fell into one electropherotype with the three remaining strains forming unique types. Strains in the large group were distinguishable by outer membrane profile into one large subgroup (17 strains) and 5 smaller groups, 2 of these containing 2 and 4 strains respectively.

Our finding that most nontypable strains form unique types is similar to the observations made by workers using enzyme electrophoretic techniques (Musser *et al.* 1986; Porras *et al.* 1986*b*) or outer membrane protein subtyping (Barenkamp, Munson & Granoff, 1982; Leob & Smith, 1980), and provides further evidence of the extreme genetic variability of *H. influenzae*. That some strains isolated from different individuals had the same polypeptide profile lends further support to the clonal nature of the population structure, and the failure to find type b and nontypable strains with the same polypeptide profile provides evidence favouring the hypothesis that the latter strains are not phenotypic variants of common serotype b clones, as already suggested by studies on restriction modification systems (Stuy, 1978) outer membrane proteins (Loeb & Smith, 1980; Barenkamp, Munson & Granoff, 1982) and enzyme electropherotyping (Musser *et al.* 1986; Porras *et al.* 1986*b*).

No correlation could be made between disease type and SDS–PAGE whole cell subtype.

Subtyping of *H. influenzae* by SDS–PAGE and Dice analysis of whole-cell polypeptides is not only more discriminatory than biotyping and outer membrane protein typing but also possesses an advantage in that a single technical manipulation provides a large number of markers, no strain in our studies so far giving fewer than 32 polypeptide bands suitable for analysis, with 39 bands being the average number given by the strains that we have examined. Thus many more markers are generated than by the techniques used in biotyping or outer membrane protein typing or are generally used in enzyme electrophoretic subtyping. The disadvantages of whole-cell polypeptide analysis are those of any polyacrylamide gel technique, the most notable being problems associated with inter-gel variability. These can largely be surmounted by the appropriate use of previously subtyped strains as markers in gels.

We have arbitrarily adopted 90% similarity as the boundary of type b clusters in polypeptide analyses. While there can be little doubt that many strains within clusters so defined are clonally related, particularly those with identical or ex-

tremely closely related polypeptide profiles, the boundaries of clusters which limit clones cannot at present be clearly delimited due to our lack of understanding of the genetic basis of variations in polypeptide mobility in the gel system we use.

Our finding that identical polypeptide profiles were shown by strains isolated from a single individual over a short period and by strains that were closely related epidemiologically indicates that whole-cell polypeptide typing is likely to be a technique suitable for establishing the epidemiological relationship of strains. Further work is planned to investigate this possibility. We also plan to extend the studies reported here to include strains from other parts of the UK. It will also be of interest to establish the relationship of British strains to those from the USA, particularly to the subclone ET-1/OMP1H/biotype 1 described by Musser and co-workers (1985) which appears to have dramatically increased in frequency in the USA between 1939–54 and 1977–80.

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