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Molecular epidemiology of recent United Kingdom isolates of *Neisseria meningitidis* serogroup C

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SUMMARY

The genomes of 34 recent United Kingdom isolates of *Neisseria meningitidis* serogroup C were examined by restriction enzyme digestion and by conventional and pulsed-field gel electrophoresis (PFGE). Strains were assigned to groups on the basis of the Dice similarity coefficient; strains with values of > 92% were considered to be clonally related. Twelve clones were identified by PFGE. Strains of two clonal groups predominated. Restriction endonuclease analyses using the 'high frequency cleavage' endonuclease *Stu* I and conventional electrophoresis gave 11 groups; in general it had lower resolving power than PFGE.

INTRODUCTION

In the United Kingdom, *Neisseria meningitidis* is the commonest cause of bacterial meningitis in children [1]. In England and Wales, the majority of cases (69%) are caused by serogroup B meningococci, with 28% of cases following infection with serogroup C strains [2]. A similar distribution has been found in Scotland; of strains isolated from CSF and blood in 1992, 61·2% belonged to serogroup B and 35% to serogroup C [3].

Most disease isolates within the serogroup B and C meningococci have been assigned by multilocus enzyme electrophoresis (MLEE) to one of three genetically distinct lineages, each containing a number of related electrophoretic types [4]. Serogroup C organisms are responsible for sporadic and endemic disease, being intermediate between genetically diverse serogroup B meningococci and the relative genetic and antigenic stability of clones of serogroup A [5, 6]. In the UK, serogroup C meningococci are also responsible for epidemics and their frequency seems to be increasing [7].

Several typing schemes have increased understanding of the epidemiology of *N. meningitidis* infections. Techniques used include serogrouping, serotyping, and serosubtyping, MLEE and electropherotyping. The results of some of these techniques have been used to support the hypothesis that *N. meningitidis* has a clonal population structure [6, 8–10]. Serological classification is phenotypically based and suffers from the deficiency that groupings based on it may contain strains that are genotypically dissimilar. MLEE indexes the genotype and has been widely used to study genetic relationships among meningococci [4, 8, 9]. It is labour intensive and time consuming, however. Although efforts have been

made to type meningococci at the DNA level by restriction endonuclease analysis (REA) [8, 11, 12] such techniques often produce complex patterns that are difficult to interpret.

Pulsed-field gel electrophoresis (PFGE) allows the analysis of large fragments produced by the digestion of DNA with rare cutter enzymes [13]. This method has been used to study the epidemiology and molecular biology of a number of bacterial species, to obtain physical maps of bacterial genomes and to determine the map locations of markers not previously mapped and/or new mutations [13–16]. It has proved to be highly discriminatory and has enabled the resolution of isolates indistinguishable by serological classification methods, antimicrobial susceptibility and ribotype. Examples of studies in which it has been found to be highly discriminatory include those on *Escherichia coli* [17], *Staphylococcus aureus* [18], *N. gonorrhoeae* [19] and *N. meningitidis* [20].

In this paper we report on the use of PFGE and REA analysis to study the genetic structure of *N. meningitidis* serogroup C recently isolated in the United Kingdom.

MATERIALS AND METHODS

Bacterial strains

Thirty-four serogroup C strains of *N. meningitidis* were obtained from the Department of Medical Microbiology Aberdeen, University of Aberdeen Medical School, Foresterhill, Aberdeen AB9 2ZD; Dr R. J. Fallon (Department of Laboratory Medicine, Ruchill Hospital, Glasgow) and Dr D. M. Jones (Public Health Laboratory, Withington Hospital, Manchester). These strains were serogrouped, serotyped and serosubtyped by Dr L. Smart (Department of Laboratory Medicine, Ruchill Hospital, Glasgow. Table 1). Isolates were stored on beads at −70 °C (Protect vials, Technical Services Consultants, Bury, Lancs, UK). Seventeen strains were first isolated from different parts of Scotland and 17 from England between 1985 and 1987. There were no known epidemiological relationships between the strains except for two strains isolated from a child and a kissing contact in Falkirk. Three non-serogroup C strains were chosen for a determination of genome size because they gave fragments which separated well when digested with *Spe* I or *Nhe* I and resolved by PFGE. They were A7 (11138/79 an A:4.21 P1.10), A9 (182987/79 an A:4.21 P1.10) and A17 (31084/87 a B:16.P1.2) all isolated from Aberdeen.

DNA extraction

DNA was extracted using a modification of the method of Pitcher and colleagues [21]. Strains were grown on Chocolate blood agar and incubated for 18 h at 37 °C in the presence of 5% CO_2. Bacteria were harvested into a microfuge tube containing 0·3 ml 10 mM-Tris (pH 8·0), 1 mM-EDTA (TE8), and lysed by the addition of 0·3 ml of 5 M guanidium thiocynate, 0·1 M-EDTA and 0·5% Sarkosyl (GES reagent) for 10 min at room temperature. 0·25 ml of 7·5 M cold ammonium acetate was added and the tubes were placed on ice for 10 min followed by the addition of 0·5 ml of chloroform-isoamyl alcohol (24:1) and the suspension mixed thoroughly and centrifuged for 20 min at 12500g. DNA was recovered from the aqueous phase by precipitation with 0·54 volumes of cold isopropanol followed by

centrifugation at 6500g for 2·5 min. Pellets were air dried and dissolved in water. Precipitation was repeated using cold absolute alcohol. Following a brief centrifugation (3 min) at 12500g, the DNA was dried and redissolved in 350 μl distilled water.

Restriction endonuclease digestion for REA analysis

Five to eight micrograms of DNA was digested with 8–10 units of *Stu* I (Pharmacia 27-0973-01, USA) according to the manufacturer's instruction and electrophoresed in 0·8% horizontal agarose gels (Sigma, No. A6877) in 89 mM-Tris, 89 mM boric acid (Sigma B7901) and 2 mM-EDTA (Sigma No. E5134) TBE buffer. After running the gel at 100 V constant voltage by BioRad DNA sub cell for 13 h the gel was stained with ethidium bromide for 20 min. A 1 Kb 'ladder' (Gibco BRL) was used as DNA size marker. The gel was washed under tap water for 40 min and pictures were taken under UV transillumination.

Preparation of genomic DNA samples for PFGE analysis

Fresh overnight cultures were harvested, washed with SB solution (10 mM Tris-HCl pH 7·5, 1 M-NaCl), resuspended in 1 ml of SB, mixed with 1 ml of low-melting point agarose (A3054 agarose, Sigma) 1·5% at 44 °C, distributed into the slots of a mould with 10 inserts of 200 μl (BioRad Laboratories) and allowed to solidify at 4 °C for 30 min. For lysis the agarose plugs were transferred into 2 ml of LB lysis solution (10 mM-Tris-HCl pH 7·5, 1 M-NaCl, 100 mM-EDTA, 0·5% 20 cetyl ether, 0·2% sodium deoxycholate, 0·5% sarkosyl, 1 mg/ml lysozyme and 20 mg/ml RNase and incubated overnight at 37 °C with gentle shaking. The inserts were then transferred into 2 ml of PB solution (0·5 M-EDTA pH 9), 1% sarkosyl and 1 mg/ml proteinase K), for 2 days at 50 °C with gentle shaking. The inserts were washed thrice with 20 ml of TE buffer (10 mM-tris-HCL pH 7·5, 0·1 mM-EDTA) containing 200 μl of 10 mM-PMSF (phenylmethyl-sulfonyl fluoride P 7626, Sigma) to inactivate enzyme activity. The inserts were then washed three times with TE buffer for 30 min and stored in TE at 4 °C.

Restriction endonuclease DNA digestion of inserts for pulsed-field gel analysis

Prepared inserts in REB ($\frac{1}{6}$ volume of insert, 125 μl distilled water, 20 μl restriction endonuclease buffer, 10 μl of 20 mM dithiothreitol and 10 μl of 2% Oxoid skimmed milk powder solution) were equilibrated overnight at 4 °C and digested 24 h at 37 °C using 20 U of endonuclease.

Pulsed-field gel electrophoresis

Digested inserts were sealed into slots in an agarose (Sigma A-9918) 1% gel and electrophoresed in a counter-clamped homogenous electric field (CHEF, DR 11 BioRad) apparatus with a hexagonal electrode array for 22 h at 200 V with 10 sec initial and final pulse times. Gels were stained with ethidium bromide solution (5 mg/ml) for 30 min and then destained in distilled water for 60 min before being photographed under UV transillumination with a Cybertech CS-1 Camera with image processing software Version 2.0 (Cybertech Bergmanstr, Berlin, Germany). CHEF DNA size standard bacteriophage lambda concatemers (Bio Rad 170-3635) were used as size standards.

Analysis

The similarity of banding patterns obtained by direct REA or PFGE was estimated by the method of Dice [22] in which % similarity = (number of shared bands/total number of bands) × 100. For Dice analysis of PFGE, all bands present in each track of the gel were used. The pattern of bands in the whole track was then compared. Similarity matrices generated from Dice coefficients were used to construct dendrograms by the average linkage method [23].

RESULTS

Selection of restriction endonuclease

Genomic DNA from *N. meningitidis* strains was digested with the low-frequency cleavage (LFC) enzymes *Nhe* I, *Spe* I, which recognize six base-pairs sequences containing the tetranucleotide CTAG, an infrequent sequence in most bacterial genomes [15]. Bygraves and Maiden [20] showed that when strains of *N. meningitidis* were analysed by PFGE, the endonuclease *Sfi* I gave the least number of fragments when compared to endonucleases *Spe* I and *Nhe* I; the latter enzymes are cheaper and showed greater pattern diversity. *Spe* I and *Nhe* I digests gave 12–22 and 8–18 fragments respectively by PFGE. Strains with similar *Spe* I patterns were also similar when digested with *Nhe* I. The reproducibility of PFGE patterns generated by both *Spe* I and *Nhe* I was examined by analysis of the same bacterial strain on several occasions and identical patterns were observed for repeated analyses as described by Bygraves and Maiden [20]. A DNA molecular marker was included on each gel to enable comparisons to be made between gels. Stability of PFGE patterns was confirmed when strain JN35 (H2146) was subjected to five subculture passages.

Of four high-frequency cleavage (HFC) enzymes (*Hin*d III, *Eco*R I and *Stu* I) used to digest meningococcal DNA, *Stu* I digestion gave the least number of fragments (23–32) with sizes that ranged from > 150 bp to 1·6 kb.

Electrophoretic karyotype (EK) patterns

Genomic DNA digested with *Spe* I generated 12 banding patterns (Table 1, Figs. 1 and 2). EK pattern 1 was given by 13 (38%) strains isolated from patients in a number of different localities in Scotland and England. Nine (26%) strains gave EK pattern 2; these strains also had a wide geographical distribution in Scotland and England. EK patterns 3 and 4 were given by 2 (6%) strains; EK pattern 3 strains were from Aberdeen and Glasgow and EK pattern 4 strains were from Aberdeen and Sheffield, 438 km distant. Strains showing EK patterns 5, 6, 7, 8, 9, 10, 11, and 12 were unique.

Analysis of the similarity indices of the strains by Dice method [20], and adopting an arbitrary limit of > 95% similarity to delimit clones, showed that EK patterns 1, 2, and 3 defined clonal groups. Strains 06407/79 and H2146 gave EK pattern 4 and are probably clonal, as they have 92% similarity.

Relationship between EK patterns and REA using Stu I

A comparison of EK patterns with those given by the 34 strains when analysed by REA showed 71% agreement (Table 1). Whereas EK pattern 1 was given by 13 strains, REA pattern 1 was given by 15 strains which included all 13 strains of

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Table 1. *Serogroup C meningococcal strains and their characteristics*

Strain*	Site†	Year of isolation‡	Type/ subtype§	Location	PFGE (karyotype pattern)	RAE (*Stu* I pattern)	Ribotype** (Ref. 11)	Sulph††
20760/87	CSF	1987	2a: P1.1,7	Aberdeen	1	1	1	S
G.Shain*	BC	1987	2a	Aberdeen	1	1	1	S
86/4387*		1986	NT	Aberdeen	1	1	1	S
87/07773*	BC	1987	2a	Aberdeen	1	1	1	R
87/07945	NS	1987	2a	Dumfries	1	1	1	R
87/03454	NS	1987	2a	Dumfries	1	1	1	R
87/10077*	KA	1987	2a	Falkirk	1	1	1	R
87/10078*	TS	1987	2a	Falkirk	1	1	1	R
87/07171	CSF	1987	2a	Fife	1	1	1	R
G1666	CSF	1986	2b	Gloucester	1	1	1	R
H2142	CSF	1987	2b	Birmingham	1	4	1	R
H2174*	CSF	1987	2a, P1.5	Lancaster	1	1	1	R
H2177	BC	1987	2b	Colchester	1	1	1	R
H578	CSF	1987	2b	Plymouth	2	2	1	S
H629	CSF	1987	2b	Plymouth	2	3	1	S
H796	CSF	1987	2b	Plymouth	2	2	1	S
5716/87	BC	1987	2b: P1.2	Aberdeen	2	2	1	S
87/08738		1987	2b: P1.2	Glasgow	2	2	1	S
85/17217	TS	1985	2b	Lanarkshire	2	2	—	S
J11	CSF	1988	2b	King's Lynn	2	2	1	S
H2111	CSF	1987	2b: P1.2	Burnley	2	3	1	S
87/09770	TS	1987	2b	Dumfries	2	2	1	S
86/18747		1986	2b: P1.10	Aberdeen	3	1	1	S
87/09120	BC	1987	2b	Glasgow	3	1	—	S
06407/79		1979	NT: P1.13	Aberdeen	4	7	—	S
H2146	BC	1987	NT: P1.15	Sheffield	4	5	—	R
44607/87	CSF	1987	15: P1.17	Aberdeen	5	8	8	S
87/08746	BC	1987	2b: P1.16	Glasgow	6	3	—	S
87/00723	CSF	1987	15: P1.16	Edinburgh	7	9	12	S
H2103	BC	1987	NT	Exeter	8	10	1	S
H2110	BC	1987	NT	Liverpool	9	11	4	S
H2145	CSF	1987	1: P1.15	Taunton	10	6	4	S
H2165	BC	1987	2a	Preston	11	1	1	R
H2178	CSF	1987	NT	Blackburn	12	11	—	S

* Strains were resistant to rifampicin.
† CSF, cerebrospinal fluid; BC, Blood cultures; NS, Nasal swab; KA, Knee aspirate; TS, Throat swab.
‡ Year of isolation.
§ NT, Non-typeable, subtypes indicated where possible, the remaining strains were not sub-typeable.
** Ribotype results obtained from reference 11.
†† S = Sensitive to Sulphadiazine. R = Resistant to Sulphadiazine.

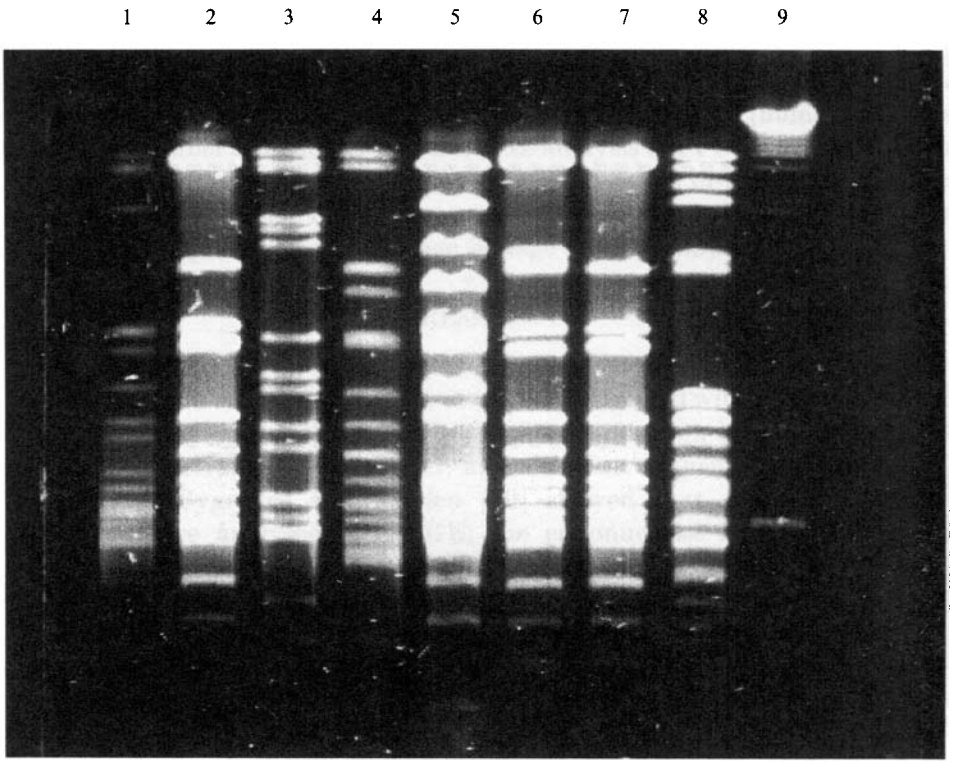

Fig. 1. PFGE of *Spe* I cleaved genomic DNAs from *N. meningitidis* serogroup C showing dissimilar profiles. Lanes: 1, H211; 2, H2142; 3, H2145; 4, H2146; 5, H2165; 6, H2174; 7, H2177; 8, H2178. Bacteriophage lambda concatemers (lane 9) were used as molecular size standard (48·5 kb).

EK pattern 1. Strains 87/09120 and 86/18747 which gave REA pattern 1 only showed 76% similarity when compared with the 13 strains by PFGE analysis; all 13 strains were very similar sharing > 95% similarity indexes when analysed by PFGE and REA. Differences between the two patterns may be due to differences between the two techniques. EK patterns were generated from PFGE analysis of the complete genomes of the strains. With the 'high frequency cleavage' endonuclease *Stu* I, not all fragments were resolved, thus REA patterns did not reflect the complete genomes of the strains. Differences between the two techniques are best shown by two dendrograms (Figs. 4*a* and *b*) which demonstrate that isolates of the serogroup C are genetically diverse and also that strains of the same serotype may not necessarily be closely related.

Determination of genomic length

Three strains 11138/79, 182987/79 and 31084/87 were each digested with *Spe* I and *Nhe* I and used to estimate genome size. The mean of the fragment size generated by *Nhe* I was $1{\cdot}9 \pm 0{\cdot}2$ Mbp and from *Spe* I fragments was $2{\cdot}3 \pm 0{\cdot}2$ Mbp. The genomic size of *N. meningitidis* was estimated to be $1{\cdot}9(\pm 0{\cdot}2) - 2{\cdot}3(\pm 0{\cdot}2)$ Mbp, similar to the 1·8–2·0 Mbp [20] and 2·3 Mbp [24] reported by other investigators.

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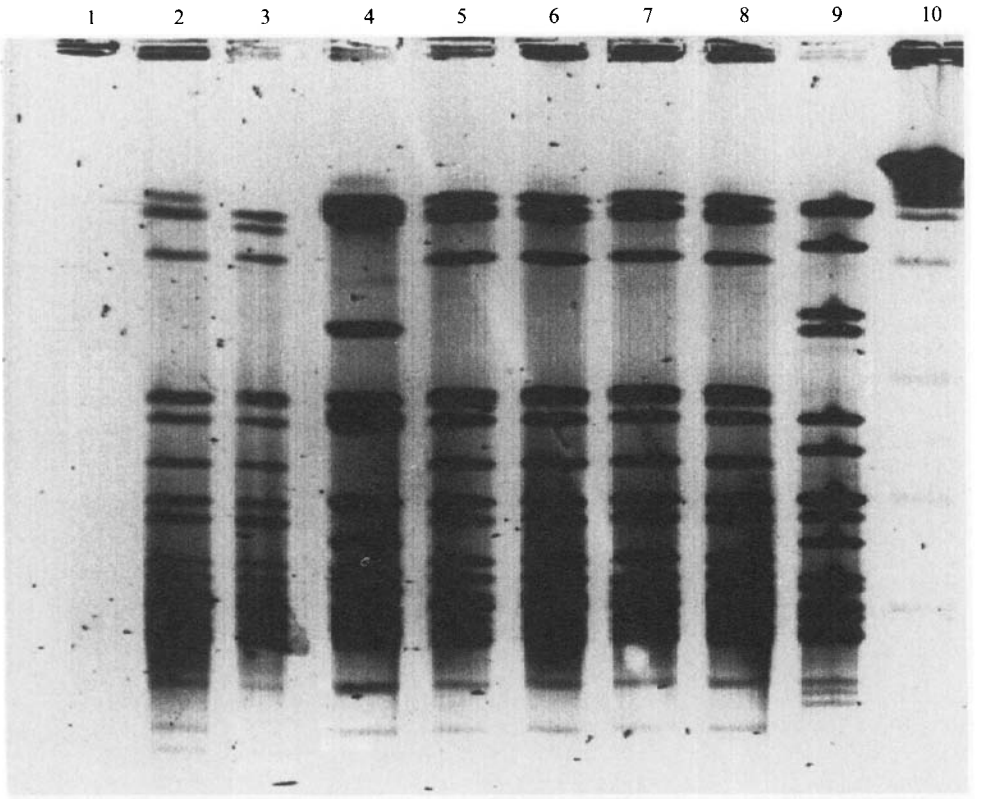

Fig. 2. PFGE of *Spe* I cleaved genomic DNAs from *N. meningitidis* serogroup C isolates showing similar profiles. Lanes: 2, 5616/87; 3, 85/17217; 5, H578; 6, H629; 7, J11; 8, H796. Lanes: 4, G1666; and 9, H2103 show dissimilar profiles. Molecular size standard (lane 10) was as Figure 1.

DISCUSSION

Analysis of our collection of disease-associated strains of meningococci by PFGE showed that two clones predominated, accounting for 64% of strains. Clone 1 was widely distributed through the United Kingdom and contained strains of serotypes 2a (69%) and 2b (31%). Serotypes 2a and 2b and subtype P1.2,5 have been associated with the electrophoretic type (ET)-37 complex which is endemic in the United Kingdom [25, 26]. Thus clone 1 characterized by C:2a may belong to electrophoretic type (ET)-37 complex. Strains in the ET-37 complex have been responsible for the majority of endemic serogroup C disease in the 1980's in the United States [27] and Italy [28]. They were the second most common isolates from cases in Canada [29] and Norway [6]. Strains belonging to this complex have been isolated world-wide during single-clone outbreaks and may form a minor part of outbreaks caused by other meningococci [26].

Clone 2 strains also had a wide distribution in the United Kingdom; this clone was made up solely of serotype 2b strains. Some members (38%) of clone 2 had the phenotype 2b:P1.2 which is characteristic of cluster MLEE A4 [4]. Recently eight serogroup C strains isolated in London and Scotland have been assigned to

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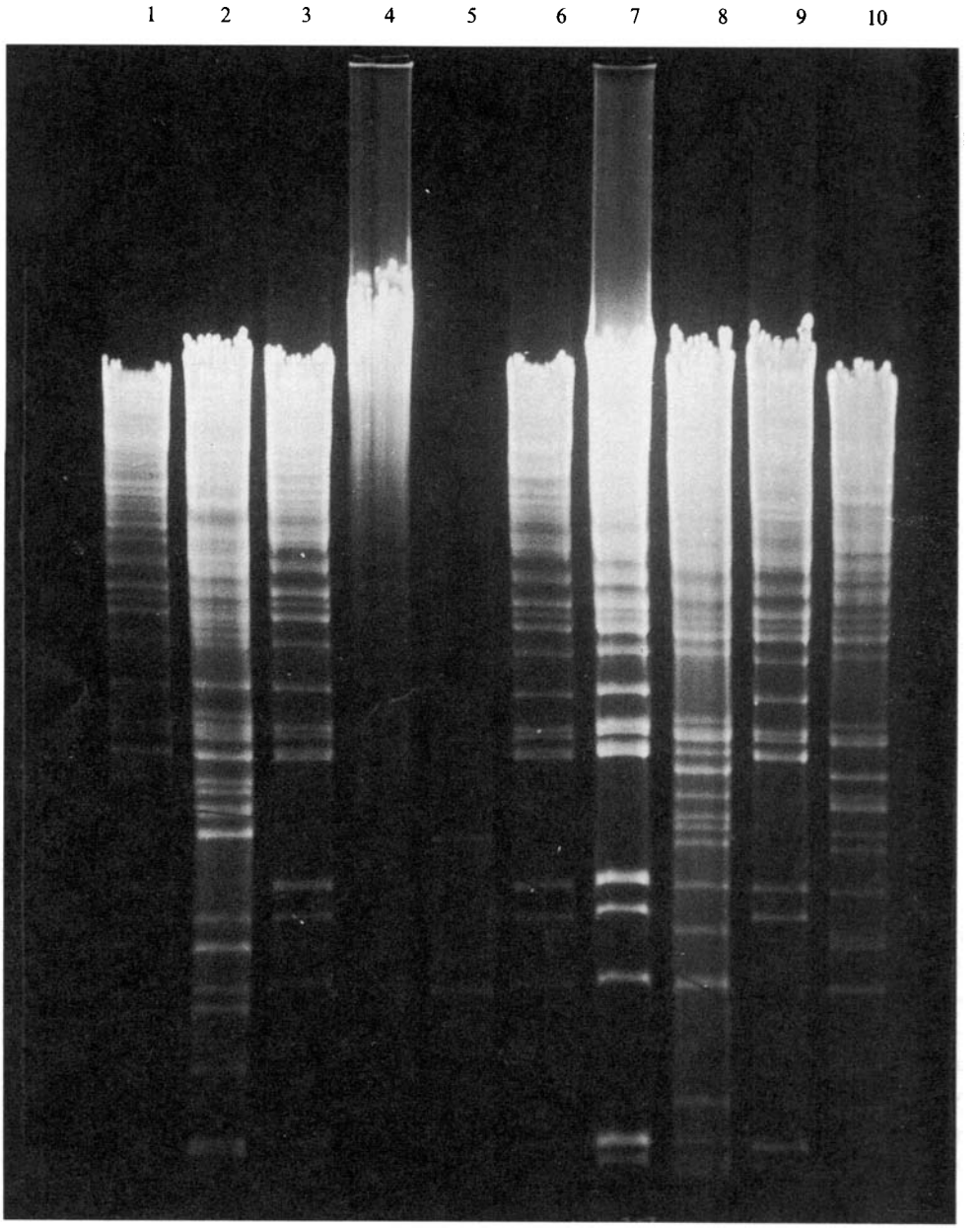

Fig. 3. Direct REA of chromosomal DNA from isolates of *N. meningitidis* after digestion with *Stu* I. Lanes: 1, 87/08738; 3, H578; 6, J11; 7, H796; 9, 85/17217 showing similar REA profiles. Lanes: 5, is 1 kb ladder, (BioRad); 2, 87/00723; 8, H2103 and 10, H2142 showing dissimilar profiles. Lane 4 did not resolve. Details of isolates are given in Table 1.

cluster A4 [25]. Other strains of serotypes 2a and 2b which are not members of the two clones (i.e. ET-37 complex or A4 cluster) also cause infection in the United Kingdom. Thus, serogroup C meningococcal strains isolated in the United Kingdom are genetically diverse. This diversity of strains of serogroup C has been

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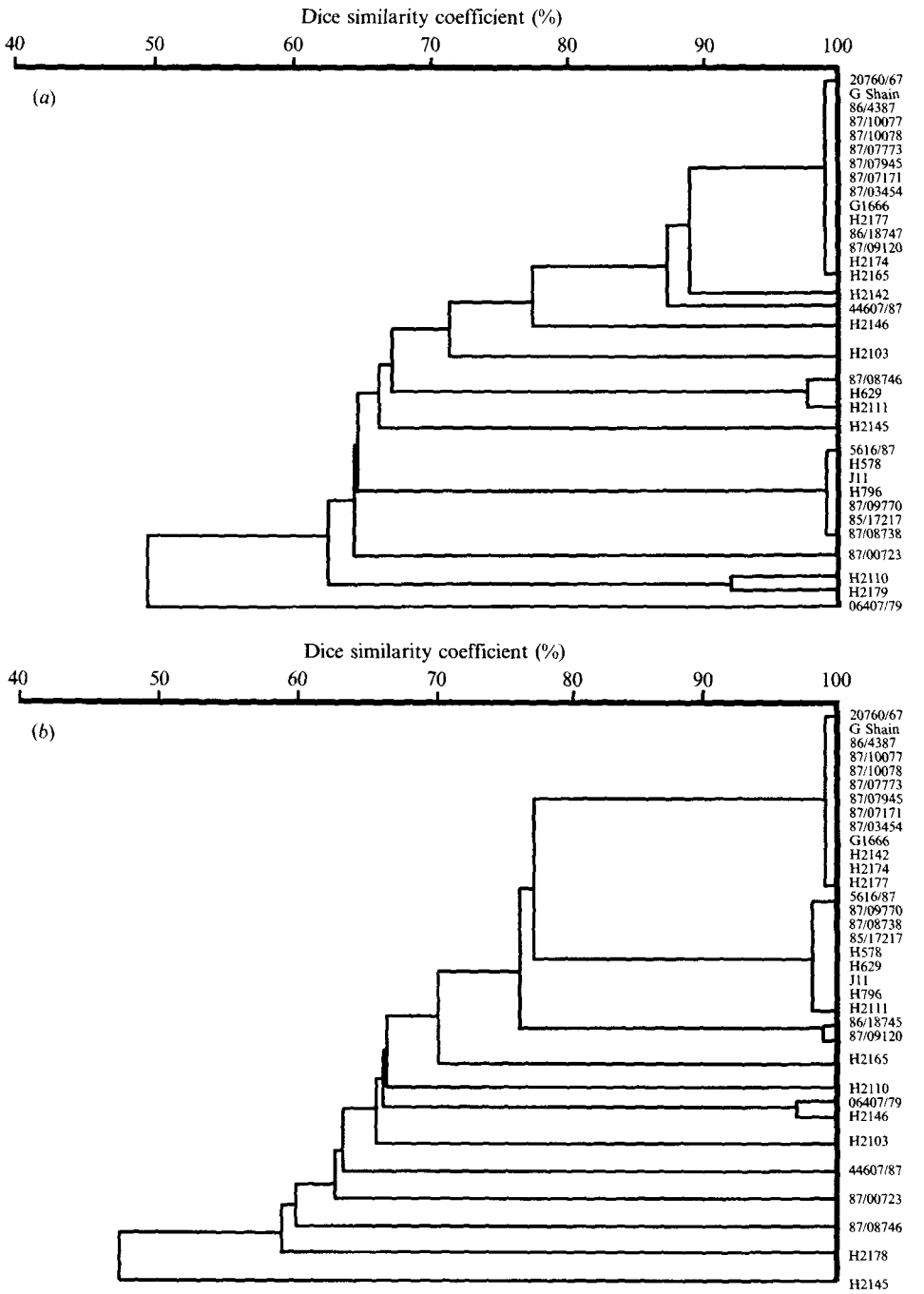

Fig. 4. Dendrograms showing the relationships between the Dice value for the dissimilar isolates, as estimated by direct REA (*a*) or PFGE analysis (*b*).

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shown elsewhere by Wang and colleagues [26], but contrasts with the relative genetic stability of clones of serogroup A [5].

Clinical management of patients suffering from meningococcal infections caused by strains of a particular clone will be the same provided the strains have the same antibiogram. However, if some members of a clone are resistant to rifampicin, use of rifampicin in chemoprophylaxis among contacts of index patients can only help to spread rifampicin-resistant strains. Six strains (46%) which belonged to clone 1 were resistant to rifampicin.

Mulligan and Arbeit [30] suggested criteria for an effective typing system of bacteria to be those of typeability, reproducibility and discriminatory power. Currently the most widely used method for identifying bacterial clones is MLEE which has been successfully applied to the study of genetic relationships among meningococcal strains [4, 31]. MLEE typing offers both the ability to discriminate and to estimate genetic relatedness among isolates. In typing meningococci, serotyping provides more specific information than serogroups, however when compared with MLEE, serotyping provided less discrimination of isolates [27]. MLEE is labour intensive, time consuming and only indexes the genotype indirectly.

Nucleic acid-based methods for subtyping bacteria have proved very useful for epidemiological investigations. These methods have high discriminating capacity, are reproducible, and do not need the use of organism-specific reagents. Several of the methods can be used for typing all strains and data generated can be evaluated objectively and analysed statistically. Furthermore, they are amenable to automation [32]. REA with high frequency cleavage endonucleases resolved by conventional polyacrylamide [8] or agarose gel [11] often produces complex patterns that are difficult to interpret, are affected by the choice of restriction endonucleases, electrophoretic conditions and system used. Typing of meningococci by REA as described by Bjorvatn and colleagues [8] requires the use of a special power pack capable of > 500 V. The polyacrylamide gels used are not as easy to manage as agarose gel and are generally more expensive than the agarose gels. Comparison of chromosomal digestion patterns has been used in epidemiologic studies of *N. meningitidis* [11]. Digestion of the chromosomal DNA with *Stu* I resolved by conventional electrophoresis correlated well with that of low-frequency cleavage with *Spe* I resolved by PFGE (Fig. 3*a* and *b*, Table 1), showing up to 71% correlation. However, PFGE gave better discriminatory capacity than REA using *Stu* I.

Ribotyping has proved to be of value in typing meningococci [11, 33]. In a study of 44 strains of *N. meningitidis* serogroup C isolated from Los Angeles, ribotyping showed a greater discriminating capacity than MLEE for subtyping serogroup-C meningococci [33]. However, ribotyping requires multiple steps, is time-consuming and labour intensive. Also for each bacterial species ribotyped, the appropriate restriction endonuclease(s) must be determined [32]. Again, characterization of 94 United Kingdom meningococcal strains by ribotyping and by REA using *Bgl* II showed that ribotyping gave lower discrimination (11). Twenty-eight of those strains which were included in this study were subtyped by PFGE into 8 subtypes whereas by ribotyping they were subtyped into only 4 ribotypes [11].

Three strains that had been ribotyped [11] were used to evaluate results of the

ribotyping method against PFGE. Strains 87/10077 (C:2a non-subtypeable) and 87/10078 (C:2a non-subtypeable) were isolated from a child and the kissing contact, his father; when analysed by PFGE, they were identical as expected. Strain 87/00723 (C:15/P1.16) gave a very different PFGE restriction profile from those of strains 87/10077 and 87/10078, in agreement with results of the ribotyping method [11; Table 1]. Though related strains showed similar restriction profiles, there was sufficient diversity among the strains to consider the comparison of *Spe* I restriction profiles as potentially valuable in the establishment of the clonal relationships between strains (Figs. 4*a* and *b*).

PFGE provides a useful method for resolution of clonal subgroups within populations of pathogenic bacteria, enabling the precise identification of disease-causing organisms and the documentation of their spread [17, 20]. The use of clonal analysis with PFGE fingerprint data offers several advantages. PFGE produces simple chromosomal restriction profiles without having to resort to probe hybridization methods. It resolves larger DNA fragments such as those greater than 40 Kb that are not efficiently resolved by conventional agarose gel electrophoresis. When applied to bacterial genome, PFGE can be used to determine genome size and to construct physical maps of bacterial chromosomes. PFGE is superior to both phenotyping and Southern blotting in discriminating between isolates [32]. A particular strain has a characteristic fingerprint that permits the analysis of the whole chromosome of the test organism, patterns of which are dependent on many chromosomal loci that are independent. The fragment numbers are only altered by the choice of restriction endonuclease used. The stability of patterns is maintained for several passages *in vitro* and for long periods of time; patterns are easily recognized and fragment sizes can be compared with those obtained from other isolates to establish intra- and inter-clonal relationships [20]. Once electrophoretic conditions are optimized for PFGE, it is a relatively simple and rapid technique. Stringent preparation of samples helps to minimize factors such as site-specific methylation in the chromosome and non-specific degradation of DNA that can affect fingerprint patterns [10].

However, use of PFGE involves extended isolation and restriction procedures of genomic DNA by the agarose plug method, use of large quantities of expensive enzymes and reagents for DNA isolation and restriction, and for the first-generation PFGE systems, the need to optimize electrophoretic conditions [32]. Use of second-generation PFGE systems which offer built-in algorithms automatically selecting the appropriate conditions for a particular separation renders PFGE a simple method for subtyping microorganisms [33].

In summary, comparison of REA using *Stu* I and restriction endonuclease analysis by PFGE using *Spe* I in this study indicates that both techniques are useful in subtyping strains of *N. meningitidis*. The two methods of examining chromosomal DNA showed close agreement; however, the use of *Spe* I fragment analysis by PFGE allowed the subdivision of REA groups generated using *Stu* I. Analyses of large genomic fragments by PFGE are easily performed when electrophoretic conditions have been optimized. They are reliable, detect considerable polymorphism, and are rapid and adaptable.

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