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Genetic Relatedness within and between Serotypes of *Streptococcus pneumoniae* from the United Kingdom: Analysis of Multilocus Enzyme Electrophoresis, Pulsed-Field Gel Electrophoresis, and Antimicrobial Resistance Patterns

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A collection of 54 isolates of invasive *Streptococcus pneumoniae* of serotypes 3 and 14 and serogroups 6, 9, 19, and 23 was investigated. Multilocus enzyme electrophoresis and pulsed-field gel electrophoresis suggested that two clones were represented in the collection, one of serotype 14 isolates, most of which were resistant to erythromycin, and one of serotype 9V isolates, in which resistance to penicillin (MIC, 1 μg/ml), cefotaxime, and co-trimoxazole was common. Among other isolates there was only a limited correlation between genetic relatedness measured by multilocus enzyme electrophoresis and expression of the same capsule type. However, isolates with highly related pulsed-field gel electrophoresis patterns always shared the same serotype and highly related allele profiles. Calculation of the index of association suggests a freely recombining population structure with epidemic spread of successful clones.

Respiratory disease has recently overtaken diarrheal disease as the most frequent cause of death among children in developing countries, with *Streptococcus pneumoniae* being one of the main pathogenic species (33). In the developed world, *S. pneumoniae* still causes serious and sometimes fatal infections in children and is one of the principle causes of bacterial meningitis (28, 32). As well as affecting the young, *S. pneumoniae* is an important cause of morbidity and mortality in the elderly; it is the most common etiological agent of community-acquired pneumonia, often resulting in hospitalization of previously healthy individuals. The recent spread of strains resistant to penicillin and other antimicrobial agents is now causing serious concern; in many parts of the world, the prevalence of resistant isolates is raising difficulties in determining the optimal treatment for suspected pneumococcal infection (11).

The population structure of a bacterial species could be expected to be clonal in nature because of the absence of genetic recombination that results from sexual reproduction. The classic studies on *Escherichia coli* by Selander and colleagues (24), who applied multilocus enzyme electrophoresis (MLEE) to the problem, revealed that species to be essentially clonal. Successful clones, in which isolates share indistinguishable alleles for up to 20 different enzymes, appear very stable and can spread throughout the world. It has since been found that in a number of species only one or a few clones are responsible for the majority of cases of particular disease syndromes (1). However, recombination resulting from transformation is a mechanism for genetic exchange in bacteria, particularly in those species that are naturally transformable. It has recently been determined that the population of *Neisseria gonorrhoeae* is freely recombining, and dominant clones are not detected (20). Comparable studies on population structure have not yet been done for *S. pneumoniae*, which is also naturally competent, except in the context of penicillin-resistant isolates, of which several different clones, some with widespread distributions, have been characterized (4, 9, 16, 19, 26, 30).

Capsular polysaccharide is an essential virulence determinant for *S. pneumoniae*, providing protection from phagocytosis; nonencapsulated strains have greatly reduced infectivity. Eighty-four different serotypes, determined by capsular polysaccharide, have been described. (The first stage of serotyping results in the assignation of a number, and then subtyping sera can be used to subdivide some of the number designations but not others. A number designation that cannot be subtyped, such as 3, is referred to as a serotype, but if subtyping is possible the number designation is referred to as a serogroup, e.g., serogroup 6, with subtypes referred to as serotype or subtype 6A, etc. The nomenclature used in the literature is inconsistent and is further complicated by the fact that subtyping is not performed in all studies.) Some serotypes are particularly associated with invasive disease; for example, serotype 14 and serogroups 6, 23, and 19 are the types most frequently isolated from children with serious infections (22). To some extent, this is directly related to the nature of the capsule: serogroup 6 capsules in particular are poorly immunogenic in children. However, the contribution of the genetic background and other virulence factors in determining the difference between invasive and noninvasive isolates is less clear. Kelly et al. (10) have demonstrated profound effects on virulence by transforming strains to different capsule types, but the genetic background of the strains was also important in the level of virulence obtained. A number of potential virulence factors have been described, including neuraminidase, pneumococcal surface protein A, hyaluronidase, and pneumolysin (3). Quantitative and qualitative variations in these factors are likely to contribute to differences in virulence between isolates.

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In the study described here we have used MLEE and pulsed-field gel electrophoresis (PFGE) of restriction fragments to examine clinical isolates of six of the most important serotypes and serogroups of pneumococci. We sought to determine whether isolates of a given serotype were more closely related to each other than to isolates of other serotypes and to investigate evidence of recombination among a population of isolates not preselected for antibiotic resistance.

MATERIALS AND METHODS

Selection of isolates and serotyping. Isolates were selected from those submitted to the Central Public Health Laboratory in 1993 and 1994. All isolates were from blood culture or cerebrospinal fluid and were from a variety of hospitals in the United Kingdom and Ireland (Table 1). The isolates were selected to represent six of the most common serotypes or serogroups among invasive isolates in the United Kingdom and were selected as much as possible from different hospitals. No selection on the basis of antimicrobial resistance was made, although antibiotic-resistant isolates are referred preferentially. Serology was performed by the staff of the Streptococcus and Diptheria Reference Unit, Central Public Health Laboratory, by slide agglutination with capsular typing sera (Statens Serum Institute, Copenhagen, Denmark). All isolates were serotyped on at least two occasions to confirm the results.

MLEE. MLEE methods were derived from those of Selander et al. (23), with guidance from T. Coffey and C. Dowson, University of Sussex, and subsequent modifications. To prepare enzyme extracts, the growth from three petri dishes of brain heart infusion agar was collected into 10 ml of Todd-Hewitt broth. The cells were pelleted by centrifugation and were resuspended in 0.2 ml of 50 mM Tris Cl–5 mM disodium EDTA (pH 8). The cells were lysed by three cycles of freezing in liquid nitrogen and then thawing. The cell debris was removed by microcentrifugation at 12,000 × *g* for 10 min, and the supernatant was stored in aliquots at −70°C.

In preliminary experiments with electrophoresis in starch gels, from two to five buffer systems were tested to separate each enzyme. Buffer F was found to give as good a resolution as any of the systems for all enzymes, and the gels were easy to handle. However, staining for alcohol dehydrogenase (ADH) was unreliable, and more consistent results were obtained in acrylamide gels.

Separation of glutamate dehydrogenase (NADP) (GD2), glucose 6-phosphate dehydrogenase (G6P), esterase (α-naphthyl acetate) (EST), phenylalanyl-leucine peptidase (PLP), carbamate kinase (CAK), adenylate kinase (ADK), hexokinase (HEX), phosphoglucose isomerase (PGI), and nucleoside phosphorylase (NSP) was performed in starch gels with buffer F of Selander et al. (23). Horizontal starch gels were prepared in a 20-cm gel tray (DNA Sub-Cell; Bio-Rad) sealed at the ends with waterproof tape. Gels consisted of 36 g of starch in 315 ml of buffer and were prepared in a microwave oven, with frequent mixing. Wicks were formed with Whatman 3MM paper. Gels were sliced horizontally on a Shandon Southern gel slicer, giving up to six slices per gel. The top slice was always discarded.

Separation of ADH was performed in 1.5-mm acrylamide gels. Acrylamide gels were prepared in a Mighty Small II apparatus (Hoefer Scientific Instruments). The resolving gel was 8% acrylamide (acrylamide-bisacrylamide [37:1]) and 0.39 M Tris Cl (pH 8.8), the stacking gel was 5% acrylamide– 0.125 M Tris Cl (pH 6.8), and the running buffer was 25 mM Tris– 0.25 M glycine (pH 8.3).

Staining for enzyme activities was performed exactly as described by Selander et al. (23), except that only 10 ml was used for the agarose overlays and the other stain reagents were applied as a 5-ml solution spread onto the surface of the gel slice rather than immersing the gel in 50 ml of stain solution. CAK was stained exactly as ADK, but with the addition of carbamyl phosphate at 2 mg/ml.

All scoring of electromorphs was performed by comparison with other isolates within the same gel. Electromorphs were numbered in order of decreasing anodal migration. Results were analyzed with the Multi-Variate Statistical Package, version 2.1 (Kovach Computing Services, Pentraeth, Wales). Similarities were calculated by using the Gower general similarity coefficient, and cluster analysis was by the unweighted pair group method with a random order of input.

PFGE. Isolates were inoculated into 10 ml of Todd-Hewitt broth and were incubated overnight. The cells were pelleted, resuspended in 0.5 ml of 10 mM Tris Cl (pH 7.6)–1 M NaCl, mixed with an equal volume of 2% pulsed-field certified agarose (Bio-Rad) at 50°C, and dispensed into plug molds. The plugs were incubated overnight in 6 mM Tris Cl (pH 7.6)–1 M NaCl–100 mM disodium EDTA–0.5% Brij-58–0.2% deoxycholate–0.5% *N*-laurylsarcosine–20 μg of RNase per ml–100 μg of lysozyme per ml at 37°C and then overnight in 0.5 M disodium EDTA–1% *N*-laurylsarcosine–50 μg of proteinase K per ml at 50°C, and the plugs were then washed three times for 30 min each time in 10 mM Tris Cl–0.1 mM disodium EDTA (pH 7.5). For digestion, the slices were incubated overnight with 10 U of *Sma*I or *Apa*I (Promega) in the recommended restriction buffer. The gels were 1% agarose (pulsed-field certified grade) in 0.5× TBE (45 mM Tris borate, 1 mM EDTA) and were run without ethidium bromide in the gel. Gels were run in a CHEF DR II apparatus (Bio-Rad) at 6 V/cm for 18 h with a switching time of 1 to 15 s. The gels were stained with 0.5 μg of ethidium bromide per ml and were destained in water for 1 h before photography.

TABLE 1. Serotype, ET, PFGE pattern types, antibiotic resistance patterns (antibiograms), and geographic origins of isolates examined in the present study

Isolate	Serotype	ET	PFGE pattern		Antibiogram[a]	County of isolation
			*Apa*I	*Sma*I		
PN93/164	3	18	1a	1a		East Sussex
PN93/751	3	16	1b	1b		Devon
PN93/1160	3	16	1b	1b		Bucks
PN93/1601	3	7	2	2		Greater Manchester
PN93/1639	3	18	1b	1c		Northants
PN93/1730	3	1	3a	3a		Lancs
PN94/261	3	2	3a	3b		Kent
PN94/431	3	1	3b	3c		South Glamorgan
PN94/822	3	16	1a	1d		Merseyside
PN93/142	6A	12	4	4		Norfolk
PN93/543	6A	23	5	5		Staffs
PN94/492	6A	5	6	6		Cleveland
PN94/361	6B	13	7	7		Northern Ireland
PN94/804	6B	16	8	8	S/T, T	Greater London
PN93/1293	6B	13	9	9	P, CX, S/T, CH, T	Surrey
PN93/1413	6B	30	10	10		Berks
PN93/110	9N	21	11a	11a		Greater London
PN93/1510	9N	21	11b	11b		Cleveland
PN93/909	9V	21	12a	12a		Avon
PN93/917	9V	20	12b	12b		Avon
PN93/1029	9V	20	12c	12c	P, CX, S/T	Eire
PN93/1454	9V	20	12d	12d	P, CX, S/T	Hants
PN93/1802	9V	20	12c	12c	P, CX, S/T	Greater London
PN94/661	9V	20	12e	12e		Greater London
PN94/812	9V	20	12c	12f	P, CX, S/T	Greater London
PN94/926	9V	20	12e	12e	P, CX, S/T	Cheshire
PN93/132	14	10	13a	13a		West Midlands
PN93/436	14	8	13b	13b		Norfolk
PN93/637	14	3	14a	14a	E	West Yorkshire
PN93/872	14	3	14a	14a	E	Devon
PN93/908	14	3	14a	14b	E	South Glamorgan
PN93/1565	14	3	14a	14a	E	Kent
PN93/1719	14	3	14b	14a	E	Dyfed
PN94/62	14	3	14a	14b	E	Worcs
PN94/153	14	4	14c	14c	E	East Sussex
PN94/336	14	4	14d	14d		West Midlands
PN94/469	14	3	14a	14a	E	Merseyside
PN94/653	14	3	14e	14a	E	West Midlands
PN93/1329	19A	11	15	15		Humberside
PN94/152	19A	25	16	16	P	Bucks
PN93/1817	19B	3	17	17		Cheshire
PN94/856	19B	24	18	18		Hereford
PN93/1791	19F	6	20	20		Avon
PN94/87	19F	3	21	21		Kent
PN94/595	19F	17	22	22	E	Gwynedd
PN93/100	23A	22	23	23		Greater London
PN93/221	23A	29	24	24		Humberside
PN93/720	23F	14	25	25	P, CX, S/T, E, CH	Surrey
PN93/1345	23F	26	26a	26a		Norfolk
PN93/1660	23F	28	26b	26b		Notts
PN94/258	23F	19	27	27		Cambs
PN94/310	23F	27	28	28		South Glamorgan
PN94/682	23F	9	29	29		Devon
PN94/1744		15	19	19		Bucks

[a] P, penicillin; CX, cefotaxime; S/T, sulfamethoxazole-trimethoprin (co-trimoxazole); E, erythromycin; CH, chloramphenicol; T, tetracycline.

TABLE 2. Allele profile for each ET

ET	No. of isolates	Allele profile[a]									
		ADH	ADK	CAK	EST	GD2	G6P	HEX	NSP	PGI	PLP
1	2	2	2	2	3	2	2	1	2	1	2
2	1	3	2	2	3	2	2	1	2	1	2
3	10	2	2	2	3	2	2	1	1	1	3
4	2	2	2	2	3	2	2	1	1	1	1
5	1	2	2	2	3	1	2	1	1	1	4
6	1	2	2	1	3	2	2	1	2	1	3
7	1	3	2	2	2	1	2	1	1	1	2
8	1	3	2	2	2	1	2	1	1	1	3
9	1	3	2	2	3	1	2	1	2	1	3
10	1	3	2	2	2	1	2	1	2	1	3
11	1	3	2	2	3	2	1	1	1	1	3
12	1	3	2	2	4	1	1	1	1	1	3
13	2	2	2	2	3	1	1	1	1	1	3
14	1	2	2	2	3	1	1	1	2	1	3
15	1	3	2	2	3	1	1	1	1	1	2
16	4	2	2	2	3	1	1	1	1	1	2
17	1	2	2	1	3	1	1	1	1	1	2
18	2	2	2	2	3	2	1	1	1	1	2
19	1	2	2	2	1	2	1	1	1	1	2
20	7	2	2	1	3	1	1	2	1	1	2
21	3	2	2	1	3	1	1	2	1	1	3
22	1	2	1	1	3	1	1	1	1	1	3
23	1	2	3	0	3	1	1	1	1	1	3
24	1	1	2	1	3	1	2	1	1	1	3
25	1	1	2	1	3	1	2	1	1	1	2
26	1	2	2	2	1	2	1	1	2	1	4
27	1	2	2	2	1	2	1	1	1	1	4
28	1	2	2	0	1	2	1	1	1	1	4
29	1	2	2	2	1	1	1	1	1	1	4
30	1	2	4	0	2	1	2	1	1	1	3

[a] Enzyme abbreviations are given in Materials and Methods.

Antimicrobial susceptibility testing. The MICs of penicillin, cefotaxime, co-trimoxazole (sulfamethoxazole plus trimethoprim [19:1]), chloramphenicol, tetracycline, and erythromycin were determined by using doubling dilutions of antibiotic in Iso-Sensitest agar with 5% lysed horse blood. The inoculum used was 10^4 CFU for all drugs except co-trimoxazole and was 10^3 CFU for co-trimoxazole. The inoculum was applied with a Denley multipoint inoculator. All plates except erythromycin plates were incubated for 24 h at 37°C in 7% CO_2 before reading; erythromycin plates were incubated in air. The Oxford *Staphylococcus* strain was included on all plates as a control; the expected susceptibilities were always obtained. For co-trimoxazole testing, the thymidine content of the medium was checked by applying a co-trimoxazole disk (23.8 μg of sulfamethoxazole, 1.2 μg of trimethoprim) to a plate seeded with *Enterococcus faecalis* NCIB12756 (supplied by R. J. Owen); the diameter of the zone of inhibition was always greater than 20 mm.

RESULTS

Selection of isolates and serotyping. Fifty-four isolates of serotypes 3 and 14 and serogroups 6, 9, 19, and 23 were selected from pneumococci submitted to the Central Public Health Laboratory in 1993 and 1994. All were from blood culture or cerebrospinal fluid and, hence, were deemed to have caused invasive disease. Serotypes 3 and 14 cannot be further typed, but typing was repeated and subtyping was performed on all isolates of serogroups 6, 9, 19, and 23. The serotype of isolate PN93/1744 could not be confirmed. Isolates are listed by serotype in Table 1.

MLEE. The results of MLEE analysis are summarized in Tables 1 and 2. All enzymes were polymorphic except for PGI, with up to four electromorphs per enzyme. Thirty different electrophoretic types (ETs) were obtained. Eight of the ETs included more than one isolate, with ET 3 being the most common, with 10 isolates. Of these eight ETs, five were associated with single serotypes, but ETs 3, 16, and 21 contained isolates of more than one serotype (Table 1, Fig. 1).

Cluster analysis was performed by the unweighted pair group method, and 10 determinations were made, using a random order of data input. Figure 1 represents a typical result. Nine clusters of one or more isolates were consistently obtained at the 75% relatedness level, except that two isolates, PN94/258 (serotype 23F) and PN94/595 (serotype 19F), were each placed in different clusters in 3 of the 10 determinations. Clusters obtained at both higher and lower levels of relatedness were much less consistent between determinations.

Analysis of population structure. The index of association (I_A) was calculated by the method of Maynard Smith et al. (15). This compares the observed variance of K, the distance between all pairs of individuals, with the expected variance if there is no linkage between alleles. A nonzero value indicates linkage disequilibrium and may provide evidence of clonality. Calculated for all isolates, I_A was 0.28 ± 0.12. If each ET was considered an individual, I_A was -0.04 ± 0.23.

PFGE. All isolates were digested with *Apa*I and *Sma*I, and the patterns obtained with each enzyme were compared. Each pattern was assigned a number. For highly related patterns (in which at least 80% of the bands were conserved between two isolates), the same number was given and was further defined as a, b, etc. Examples of the patterns obtained with *Apa*I are shown in Fig. 2, and the results are summarized in Table 1. The results obtained with the two enzymes were very similar, although the classification of highly related patterns into subtypes differed for some isolates.

All isolates with highly related PFGE patterns had the same serotype and had ETs that differed at not more than one locus (except for isolates PN93/1345 and PN93/1660, which had different alleles at one locus, and isolate PN93/1660 had a null allele at a second locus).

Antimicrobial susceptibility. Nineteen of the 54 isolates were resistant to one or more of the antimicrobial agents tested (Table 1). Resistance was defined as an MIC of 0.1 to 1 μg/ml for intermediate-level resistance and an MIC of ≥2 μg/ml for high-level resistance for penicillin, ≥20 μg/ml for co-trimoxazole (trimethoprim-sulfamethoxazole [1:19]), ≥8 μg/ml for tetracycline, ≥8 μg/ml for chloramphenicol, and ≥1 μg/ml for erythromycin, as recommended by Klugman (11). Resistance to cefotaxime was taken as an MIC of 1 μg/ml for intermediate-level resistance and an MIC of ≥2 μg/ml for high-level resistance. Two patterns of resistance were common to several isolates, while other patterns were unique. Resistance to penicillin (intermediate level), cefotaxime (intermediate level), and co-trimoxazole was detected in five isolates, all falling within the cluster of eight serotype 9V isolates that were related by MLEE and PFGE types. Resistance to erythromycin alone (MIC, 4 to 8 μg/ml) was found in 10 isolates, 9 of which were serotype 14 and were highly related by MLEE and PFGE and the other of which was serotype 19F. Two isolates, isolates PN93/1293 and PN93/720, were multiply resistant and unique. No isolates had high-level resistance to penicillin or cefotaxime.

Genetic relatedness within serogroups and serotypes. (i) Serotype 14. Of the 12 serotype 14 isolates, 10 were closely related by both MLEE and PFGE results. All but one member of this group were resistant to erythromycin. The other two isolates of serotype 14 were related to each other, but they were genetically distinct from the major group. The serotype 14 isolates originated from widely dispersed locations (Table 1), and there was no evidence of any epidemiological link between them.

(ii) Serogroup 9. Isolates of serogroup 9 formed a cluster of isolates by MLEE, which was further divided into two related

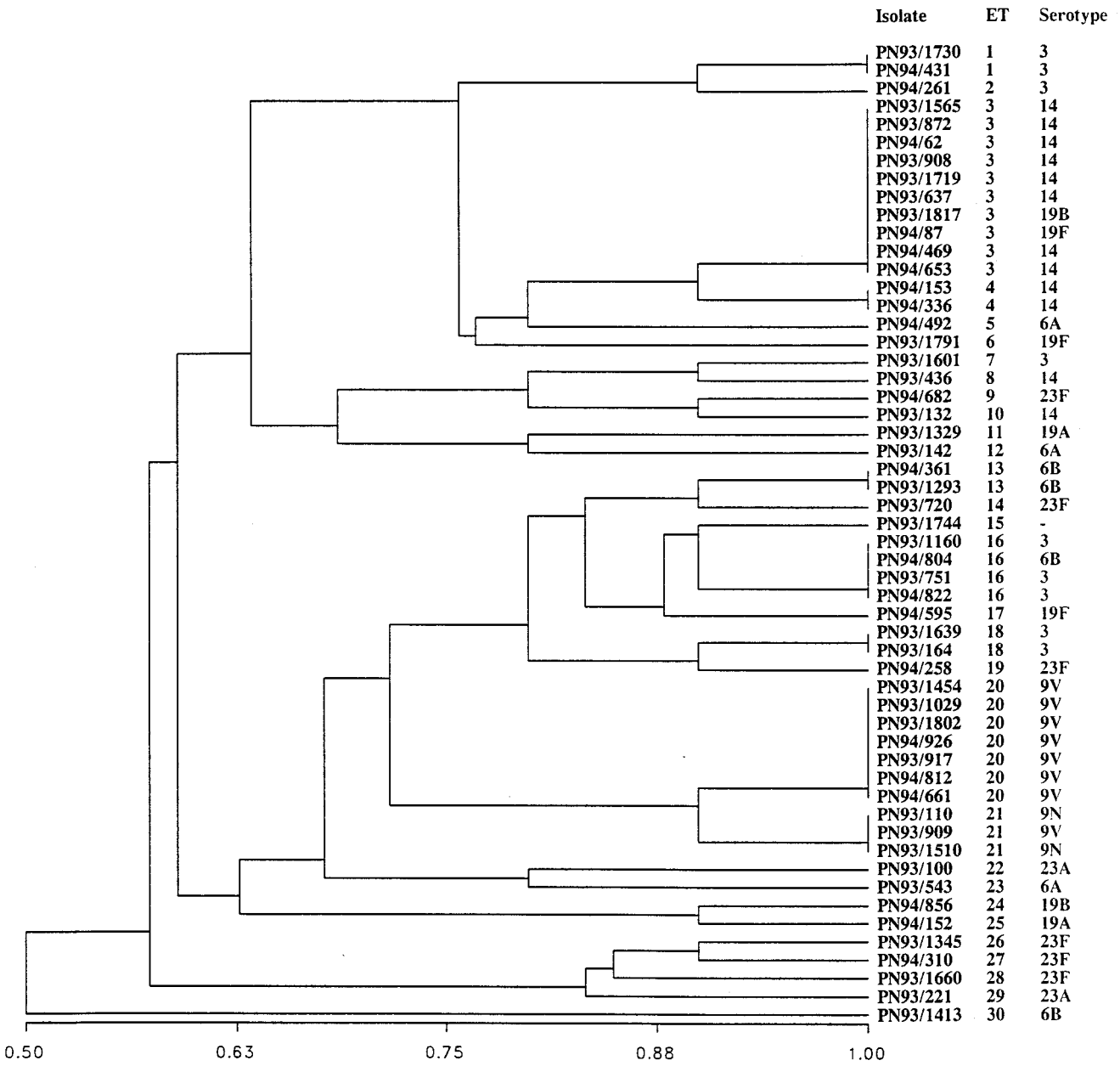

FIG. 1. Dendrogram of isolates on the basis of the MLEE results clustered by the unweighted pair group method.

ETs that corresponded for all but one isolate to the two subtypes 9V and 9N. The PFGE patterns correlated with the two subtypes. There was also an association of serotype 9V with resistance to penicillin, cefotaxime, and co-trimoxazole. Although three subtype 9V isolates originated from greater London, they were from hospitals in different parts of the city, and any direct epidemiological link between them is unlikely.

(iii) Serotype 3. MLEE divided serotype 3 isolates into three genetically distinct groups of one, three, and five isolates, respectively. The same groups were identified by PFGE with both *Apa*I and *Sma*I.

(iv) Serogroup 23. A group of four serogroup 23 isolates clustered together by MLEE, but the remaining four appeared to be genetically diverse. The four isolates clustered included two of subtype 23F that were also highly related by PFGE.

(v) Serogroups 6 and 19. Isolates of both serogroups 6 and 19 were dispersed widely through the clusters defined by MLEE. Each subtype was represented by few isolates, but even isolates of the same subtype were mostly distantly related by MLEE. All isolates in these serogroups had unique PFGE patterns.

DISCUSSION

Interpretation of MLEE and PFGE results. MLEE is generally accepted as the method of choice for measuring genetic relatedness between isolates. It measures variation that is assumed to be selectively neutral and to reflect the genotype rather than the phenotype, and it samples a number of independent loci that should be representative of the whole ge-

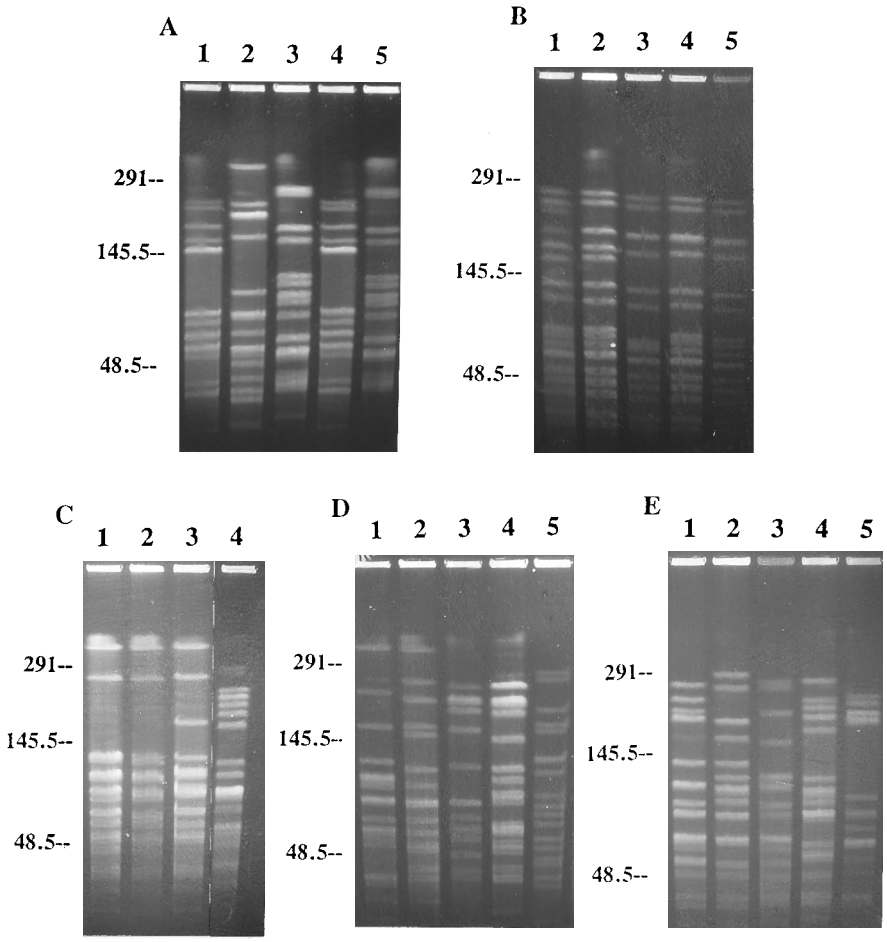

FIG. 2. PFGE patterns of *Apa*I digests from a selection of isolates of different serotypes. (A) Serotype 3 isolates. Lanes: 1, PN93/1639; 2, PN93/1601; 3, PN93/1730; 4, PN93/1160; 5, PN94/261. (B) Serogroup 9 isolates. Lanes: 1, PN94/926; 2, PN94/661; 3, PN93/917; 4, PN93/1029; 5, PN93/1802. (C) Serotype 14 isolates. Lanes: 1, PN93/908; 2, PN93/1565; 3, PN94/153; 4, PN93/132. (D) Serogroup 19 isolates. Lanes: 1, PN93/1817; 2, PN94/87; 3, PN94/856; 4, PN93/1791; 5, PN94/595. (E) Serogroup 23 isolates. Lanes: 1, PN94/310; 2, PN93/221; 3, PN94/258; 4, PN93/720; 5, PN93/100. The positions of DNA markers of 48.5, 145.5, and 291 kb are indicated.

nome. Genetic relatedness is then expressed as the proportion of loci at which two isolates are indistinguishable. Restriction fragment length polymorphism, by PFGE or conventional electrophoresis, has also been used widely to examine the relatedness of bacterial isolates including *S. pneumoniae* (8, 13, 27, 31). Highly related isolates can be recognized as having restriction fragment patterns that differ in a small proportion of bands, but when the patterns are less similar, there are important problems in interpreting levels of relatedness. Furthermore, a significant part of the variability seen may be due to genetic rearrangements other than point mutations (7).

Clones detected among U.K. *S. pneumoniae* isolates. (i) Serotype 14. In the collection of isolates assembled for the present study, two major groups of highly related isolates were detected. The first group consisted of 10 serotype 14 isolates with highly related PFGE patterns that belonged to ET 3 or ET 4 (ETs 3 and 4 differ at only one locus). We consider this to constitute a clone, in other words, a group of isolates with a common ancestor. Nine of the isolates were resistant to erythromycin. Two serogroup 19 isolates also fell into ET 3, but because they did not have related PFGE patterns, they were not considered to belong to this clone.

In a survey of bacterial meningitis in the North East Thames Region of England, 11 of 65 (17%) characterized pneumococcal isolates and 8 of 28 (29%) isolates from children were serotype 14 (29). All but two of those isolates were indistinguishable from each other by ribotype and were resistant to erythromycin; PFGE of three examples revealed patterns highly related to serotype 14 patterns in the present study (unpublished data). Taken together, these results suggest that a clone of serotype 14 is causing a considerable proportion of pneumococcal disease in the United Kingdom at the present time. The serotype 14 capsule is poorly immunogenic in young children, and numerous studies around the world have found isolates of serotype 14 to be among the most frequent causes of pneumococcal disease in this age group (17, 21, 22, 25). That the clone described here is associated with erythromycin resistance is likely to be significant to its spread. Erythromycin and, increasingly, azithromycin (cross-resistance was found in all isolates in the present study [14]) are used widely for the treatment of respiratory infections in general practice. The proportion of pneumococci resistant to erythromycin increased significantly between 1989 (3.3%) and 1992 (8.6%) in England and Wales (2), although the frequency of serotype 14 isolates among these resistant isolates is unknown. The contribution that virulence factors other than capsule could make to the success of the clone has not yet been investigated. It will be of

interest to determine whether members of the same clone are detected outside the United Kingdom.

(ii) Serotype 9V. A second group of isolates that we consider to constitute a clone comprises seven serotype 9V isolates with identical MLEE types and highly related PFGE patterns. For five of these isolates, penicillin MICs were 1 μg/ml and cefotaxime MICs were 1 μg/ml, and the isolates were resistant to co-trimoxazole, while the other two isolates were fully susceptible. A further, susceptible, serotype 9V isolate differed only at one locus by MLEE and was highly related to the others by PFGE.

A clone of penicillin-resistant serotype 9V pneumococci (MICs, generally 1 to 2 μg/ml) is known to be prevalent in Spain (26) and France (6, 12), and a U.K. isolate of the same clone has also been described (4). Penicillin-binding protein genes were indistinguishable in those isolates tested and were also identical to those in a clone of penicillin-resistant serotype 23F pneumococci found in Spain and elsewhere (4). Those isolates tested were resistant to trimethoprim (6) and co-trimoxazole (12) (the isolates in the present study highly resistant to co-trimoxazole were also resistant to trimethoprim alone [14]). PFGE of Spanish isolate 665 (kindly provided by C. Dowson [4]) reveals a pattern highly related to those of the serotype 9V isolates examined in the present study (unpublished data), suggesting that the clone of serotype 9V recognized in the present study is the same as the penicillin-resistant clone already reported. Interestingly, susceptible serotype 9V isolates in the present study were members of the same clone. Whether the susceptible isolates represent a parental clone, one or more members of which have been transformed with altered penicillin-binding protein genes, or whether they represent isolates in which resistance has been lost through transformation or other mechanisms cannot be determined at this time. However, it is notable that the full spectrum of resistance appears to be gained or lost simultaneously. Similar observations have recently been reported for serotype 9V isolates in France (12).

Genetic relatedness between serogroups and serotypes. In the present study, most of the clusters defined at the level of 75% similarity by MLEE, and even some individual ETs, contain isolates of several serotypes and serogroups (Fig. 1). In other words, with the exception of the clones mentioned above, isolates of the same serotype do not appear to be more closely related to each other than to isolates of different serotypes. This suggests either that serotype is not closely linked to the genetic lineage of the isolate or that the population is not truly clonal in structure, or both. Maynard Smith et al. (15) have described the use of an I_A to determine whether bacterial population structures are clonal or freely recombining. A value of I_A that differs significantly from zero indicates a population in which recombination is restricted, i.e., a clonal population structure. In the present study, the I_A calculated for all isolates was equivocal, with a barely significant deviation from that for a freely recombining population. When calculated on ETs, the I_A is effectively zero. This result suggests that *S. pneumoniae* has an epidemic population structure: the population as a whole appears to be freely recombining, but occasionally, highly successful strains arise and spread rapidly to produce recognizable clones. In the case of a freely recombining population, the dendrogram of MLEE results does not meaningfully represent the genetic lineages of the individual isolates (15). This may explain why some isolates with unrelated PFGE patterns and different serotypes are indistinguishable by MLEE: the frequency of a particular combination of alleles in a panmictic population is a product of the frequencies of each of these alleles in the population, not necessarily a reflection of a common ancestor.

Thus, the population structure of *S. pneumoniae* shares features in common with those of *N. gonorrhoeae*, which was found to be nonclonal (20), and *Neisseria meningitidis*, which has an epidemic population structure (15). It is probably significant that these three species are all naturally transformable, although *Haemophilus influenzae*, which is also transformable, is strongly clonal by the same method of analysis (15).

The results of other studies on the epidemiology of *S. pneumoniae* and the spread of penicillin-resistant strains fit well with an epidemic population structure in which recombination is frequent. Investigation of isolates not specifically selected for antibiotic resistance has tended to show little correlation between the typing method used and serotype: isolates of the same serotype are often diverse (8, 26, 27). Frequent recombination could cause a rapid reassortment of any markers examined, be they restriction sites, primer binding sites used for PCR, or loci used in MLEE. Recombination could also result in a change of serotype (4, 16). Hence, such studies support the notion of a freely recombining population structure. On the other hand, a number of clones of highly penicillin resistant isolates, usually also resistant to other antibiotics, have been found to have spread between different countries. For example, a serotype 23F clone has been found in Spain and the United States (18), a serotype 6B clone has been found in Spain, Iceland, Alaska, and Texas (27, 30), and a serotype 19A clone has been found in Hungary and the Czech Republic (5). This could be considered the epidemic spread of such clones, which are clearly at a selective advantage in environments with high antibiotic usage. However, it appears that penicillin resistance has arisen independently many times, and most collections of resistant pneumococci include isolates that are not closely related, again giving an indication of the high frequency of transformation possible in this species.

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