Relatedness of penicillin-resistant *Streptococcus pneumoniae* serogroup 9 strains from France and Spain

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Pulsed-field gel electrophoresis of the genomic DNA of penicillin-resistant strains of *Streptococcus pneumoniae* was carried out. Eleven clinical strains of serogroup 9 from different French towns and Paris hospitals were tested. The restriction enzymes *Apal* and *SmaI* were used to digest intact chromosomes, and the fragments were resolved by field-inversion gel electrophoresis (FIGE). Five strains were similar using *Apal* and *SmaI*. Four others were closely related when using *Apal*, and five others were closely related when using *SmaI*. These results suggest that 10 of these strains are genetically related and have a clonal origin. The profile of the eleventh strain was completely different. Thus, in a given serotype the spreading of penicillin resistance can result from both clonal and independent events. Five strains had similar FIGE profiles to strains first isolated in Spain, suggesting that a resistant strain had spread from Spain to France.

Keywords: *Streptococcus pneumoniae*, fingerprinting, FIGE, penicillin resistance, serotype

INTRODUCTION

Emergence of penicillin-resistant pneumococcal strains is a major problem in antibiotherapy. Molecular methods are needed to determine the genetic relatedness of these strains. Fragmentation of *Streptococcus pneumoniae* genomic DNA with low-frequency-cleavage restriction endonucleases and separation of the fragments by field-inversion gel electrophoresis (FIGE) provides a DNA-fingerprint of strains. It has already been shown that FIGE profiles of independent strains are different whereas profiles of clinical isolates originating from a common ancestor are similar (Lefèvre *et al.*, 1993). This typing method therefore seems suitable for testing whether several penicillin-resistant strains emerged independently or resulted from the spreading of one clone (Soares *et al.*, 1993; Versalovic *et al.*, 1993). Although more than 80 serotypes are known in *S. pneumoniae* most of the penicillin-resistant isolates belong to only a few serotypes, mainly 4, 6, 9, 14, 19 and 23 (Muñoz *et al.*, 1991, 1992; Geslin *et al.*, 1992). However, penicillin resistance is preferentially associated with certain serotypes depending upon the country of origin; serotypes 6 and 19 are mainly

found in South Africa, serotypes 2, 3, 6 and 14 are mainly found in Spain, France and Israel (Klugman, 1990), and serotypes 6, 9, 19 and 23 have been reported in a Barcelona hospital (Linares *et al.*, 1992), and in other locations in Spain together with serotype 14 (Fenoll *et al.*, 1991). The first serotype 9 strain resistant to penicillin was isolated in Spain in 1987 (Hakenbeck *et al.*, 1991). Three other strains belonging to the same serotype were later isolated in the same country (Hakenbeck *et al.*, 1991). They are resistant to similar penicillin concentrations and share the same multilocus enzyme electrophoresis (MLEE) pattern, showing that they had very likely originated from a unique clone (Sibold *et al.*, 1992). In France, the first *S. pneumoniae* strain of serotype 9 bearing resistance to penicillin was isolated in 1988, and a rapid increase in penicillin resistance in other strains belonging to this serotype has been observed recently. The frequency of penicillin resistance in serotype 9 changed from 1.6% in 1988 to 15% in 1993 (Geslin *et al.*, 1993). We have focused our investigation on serotype 9.

METHODS

Bacterial strains and growth conditions. Bacterial strains and their relevant properties are listed in Table 1. Eleven French clinical isolates of penicillin-resistant *S. pneumoniae* were studied and compared with two Spanish strains. R6 (801) is a clone of

Abbreviations: FIGE, field-inversion gel electrophoresis; MLEE, multilocus enzyme electrophoresis; PBPs, penicillin-binding proteins.

the Rockefeller University Laboratory strain R36A. Strain R36A is itself a subclone of a type 2 strain which has lost its capsule (Avery *et al.*, 1944). Strain 801 was generated from strain R6 by DNA induced transformation (Lefèvre *et al.*, 1979). The culture media have been described elsewhere (Claverys *et al.*, 1980). Cultures were incubated at 37 °C without aeration, the pH was checked at OD₅₆₀ 0.3 and NaOH was added to stabilize the pH at 7.8.

DNA preparation and restriction enzyme digestion. *S. pneumoniae* DNA embedded in agarose blocks was prepared by a method derived from that described previously for *Haemophilus influenzae* (Kauc *et al.*, 1989). Cell cultures were grown to an OD₅₆₀ of 0.3–0.5, centrifuged at 5000 g for 10 min at 0 °C, and washed with 1 M NaCl and 10 mM Tris/HCl (pH 8.0). The cells were resuspended in the same buffer and adjusted to an OD₅₆₀ of 6. The bacterial suspension was warmed to 42–45 °C and mixed with an equal volume of 1% low-melting-point agarose at the same temperature. The molten mixture was poured into Plexiglass moulds (100 µl). After solidification, the cells embedded in agarose were lysed by addition of 0.1 M EDTA, 10 mM Tris/HCl, 0.5% Brij, 0.2% deoxycholate, 0.5% Sarkosyl and incubated for 2 h at 37 °C. The agarose blocks were transferred to an equal volume of 0.25 M EDTA, 1% Sarkosyl, 100 µg proteinase K ml⁻¹ and incubated overnight at 50 °C. They were suspended in TE buffer [10 mM Tris/HCl (pH 8.0), 1 mM EDTA] containing 1 mM PMSF, incubated at room temperature for 1–2 h with gentle mixing, and washed three times with TE buffer.

For digestion of DNA, the agarose blocks were equilibrated with 1× restriction buffer. Each block was treated with 25 U restriction enzyme (New England Biolabs or BRL) in 25 µl buffer. The blocks were incubated with *Sma*I or *Apa*I for 2 h, transferred to 100 mM EDTA, and placed on ice for 15 min. Before being loaded onto the gel, the blocks were melted at 62 °C for 10–15 min.

FIGE of macrorestriction fragments. FIGE was performed in 1% (w/v) agarose for 20 h at 8 °C at 5 V cm⁻¹ in a 0.5× solution of Tris/borate/EDTA. Field inversion was supplied by a ramp (programmable power inverter; model PPI 200, MJ Research) that controls the time for the forward and reverse cycles. Programme A was used for 0.15–12.03 s in the forward and 0.05–4.01 s in the reverse direction. Programme B was used for 0.3–30 s in the forward and 0.1–10 s in the reverse direction. We commonly used 16 h of programme A followed by 4 h of programme B.

MIC determination. Exponentially growing cultures were diluted in C-medium. Bacteria were incubated in medium containing twofold serial dilutions of penicillin at 37 °C. The MIC was defined as the lowest antibiotic concentration that prevented visible growth (Laible & Hakenbeck, 1987).

RESULTS AND DISCUSSION

To determine the genetic relationships of French isolates of *S. pneumoniae* we examined 11 strains from serogroup 9 isolated in different hospitals. These strains and their characteristics are listed in Table 1, which also includes two strains from serotype 9 that were isolated independently in Spain. Digestion with *Sma*I or *Apa*I produced about 20 fragments. The FIGE program used resolved 12–15 fragments well. When the DNA from each of these strains was compared, many of these fragments

appeared to migrate to the same electrophoretic position (Fig. 1). To compare these strains we chose strain 19590 as the reference. The degree (percentage) of homology among the strains is given by the ratio of the number of fragments that are identical to the reference strain to the total number of fragments (Table 2). *Sma*I and *Apa*I restriction patterns of five French strains, 19590, 17700, 17365, 17534 and 17316, were identical both to each other and to the Spanish strain 676. The *Apa*I profile of strain 17114 and the *Sma*I profile of strains 17438 and 665 were also identical to the reference. The *Apa*I profiles of strains 17438 and 18044 were identical but differed from the reference profile by one 55 kb band which disappeared. A possible explanation is that one site had been destroyed so that the corresponding fragment co-migrated with a larger fragment, which would mean that strains 17114, 17438 and 18044 are very closely related to the reference strain; it is noteworthy that all of them are resistant to trimethoprim. Although homology between the *Sma*I and *Apa*I profiles of strains 18997 and 16415 was reduced, many bands were shared with the reference profile. Compared with the reference strain more mutations at restriction sites were evident. Therefore we suggest that strain 16415 also belongs to this family but has diverged more than the others. The profile of the last strain, 14493, was completely different from those of all the other strains (Fig. 1, Table 2). Likewise the profile of strain 801 is very different. This is not surprising since this strain is derived directly from strain R6 and shares the same restriction profile (data not shown). R6 is itself a non-capsulated type 2 strain, suggesting an independent origin from type 9 strains. The 10 most homologous strains are therefore members of the Spanish penicillin-resistant serotype 9 group that were isolated in 1988 in Barcelona (Sibold *et al.*, 1992). These Spanish strains belong to a group of four strains from serogroup 9 that are penicillin-resistant and derived from a unique clone. The first strain of this group was isolated in 1987. They differ from each other only in minor properties. The Spanish strains assigned to two distinct clonal lineages apparently harbour identical mutant penicillin-binding proteins (PBPs), 1A-2B and 2X. Mutant PBPs could thus have been transferred from a penicillin-resistant serotype 23 into a penicillin-susceptible serotype 9 or *vice versa* (Coffey *et al.*, 1991). This strongly indicates that the 10 French strains discussed above are also members of this serotype 9V group. This conclusion was also reached by comparison of PBP profiles and by MLEE analysis of 9V strains from both countries (Hakenbeck *et al.*, 1994). It is quite possible that the first strain isolated in 1987 spread in Spain and then in France. Genetic events such as mutations (Hakenbeck *et al.*, 1991; Seroude *et al.*, 1993) and/or horizontal exchanges must have occurred in at least two different lineages belonging to serogroup 9 to account for the 11 French penicillin-resistant strains investigated in this work.

These results show that the penicillin resistance that has recently emerged in France is not entirely due to the spreading of a single Spanish clone, but is also the result of independent events. Similar conclusions were drawn

Table 1. Characteristics of *S. pneumoniae* clinical isolates

The first 11 French isolates were kindly donated by P. Geslin (Centre National de Référence des Pneumocoques, Créteil, France). For the French strains, the town of isolation or the hospital (in parentheses) in Paris is shown. The two Spanish strains 665 and 676 were a generous gift of R. Hakenbeck (Max Planck Institute, Berlin, Germany). Resistance markers: Pe, penicillin; Tp, trimethoprim; Em, erythromycin; Cm, chloramphenicol. NT, Non-typable rough strain.

Strain	Origin	Isolation date	Serotype	Antibiotic resistance markers	MIC of Pe ($\mu\text{g ml}^{-1}$)
19590	Le Mans	10/1992	9V	Pe Tp	0.5
17700	Paris (Debré)	1/1992	9V	Pe Tp	4
17365	Tours	1/1992	9V	Pe Tp	1
17534	Paris (Bichat)	2/1992	9V	Pe Tp	4
17316	Paris (St Antoine)	1/1992	9V	Pe Tp	2
17114	Créteil	1/1992	9V	Pe Tp	1
17438	Brive	1/1992	9V	Pe Tp	2
18044	Brive	3/1992	9V	Pe Tp	0.5
18997	Paris (St Joseph)	8/1992	9V	Pe Tp Em Cm	2
16415	Aulnay sous Bois	11/1991	9V	Pe Tp Em	0.5
14493	Compiègne	2/1991	9N	Pe Tp	0.125
665	Spain	1988	9V	Pe Tp	1
676	Spain	1988	9	Pe Tp	1
R6 (801)	USA	1947	NT	Susceptible	0.05

Fig. 1. FIGE separation of *Apal* (a) and *SmaI* (b) restriction fragments of *S. pneumoniae* strains from serogroup 9 and from strain 801. A λ ladder is included in the last lane. The marker sizes are 48.5, 97, 145.5, 194, 242.5 and 291 kb.

Table 2. Comparison between FIGE profiles of *Apal* and *SmaI* restriction fragments

Strain 19590 is the reference strain. Percentage homology is the ratio of the number of restriction fragments that co-migrated with the reference to the total number of bands.

Strain	No. of co-migrating <i>Apal</i> fragments	Percentage homology	No. of co-migrating <i>SmaI</i> fragments	Percentage homology
19590	13	100	14	100
17700	13	100	14	100
17365	13	100	14	100
17534	13	100	14	100
17316	13	100	14	100
17114	13	100	12	85
17438	12	92	14	100
18044	12	92	11	78
18997	11	92	9	64
16415	9	69	9	64
14493	3	23	4	28
676	13	100	14	100
665	12	92	14	100
801	6	46	5	35

by MLEE analysis of resistant strains belonging to several serogroups (Sibold *et al.*, 1992). Fingerprints of a large number of isolates will be required to determine more precisely how many independent clones participated in the emergence of penicillin-resistant strains from serogroup 9 in France and in other countries.

It is notable that within this group of 13 related strains, penicillin resistance varied by up to eightfold. This might mean that resistance genes had accumulated in a parental clone resistant to a low concentration of penicillin and were then selected by antibiotic treatments that vary from hospital to hospital. Of course it cannot be excluded that the first clone was a highly resistant one and that one or several of its resistance determinants had been transferred into other genetic lineages (Coffey *et al.*, 1991). Another possibility could be reversions from high-level to low-level resistance, as we have observed in laboratory experiments (unpublished data). In hospital strains, multiple nucleotide changes do occur in penicillin-susceptible and -resistant bacteria. However, a point mutation might play a major role in modifying the level of resistance, an event that could be reversed. Answers to these questions must await a detailed analysis of the penicillin-resistance determinants of several strains sharing similar FIGE patterns. The rapid spreading of pneumococcal clones over large distances that also has been reported for other serotypes (Hakenbeck *et al.*, 1994; Muñoz *et al.*, 1991; Soares *et al.*, 1993) raises the question of the nature of the carrier of these bacteria and the mode of transmission. The ability to trace a clone by DNA fingerprinting should facilitate our understanding of these processes.

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