Multiply antibiotic-resistant *Streptococcus pneumoniae* recovered from Spanish hospitals (1988–1994): novel major clones of serotypes 14, 19F and 15F

Tracey J. Coffey,¹ Sonsoles Berrón,^{1,2} Maggie Daniels,¹
M. Eugenia Garcia-Leoni,³ Emilia Cercenado,³
Emilio Bouza,³ Asuncion Fenoll² and Brian G. Spratt¹

Author for correspondence: Brian G. Spratt. Tel: +44 1273 678309. Fax: +44 1273 678433.
e-mail: b.g.spratt@sussex.ac.uk

¹ Molecular Microbiology Group, School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK

² Servicio de Bacteriología, Centro Nacional de Microbiología, Instituto Carlos III, 28220 Majadahonda, Madrid, Spain

³ Servicio de Microbiología Clínica, Hospital General Gregorio Marañón, Dr Esquerdo 46, 28007, Madrid, Spain

We analysed a collection of 95 multiply antibiotic-resistant pneumococci, recovered since 1988 from 14 Spanish hospitals, that have MICs ≥ 0.25 μg benzylpenicillin ml^{-1} . The majority of the isolates were of serogroups 14, 23, 6, 19 and 15, which are currently the serogroups mainly associated with multiresistance in Spain. All of the serogroup 23 isolates were members of the major Spanish serotype 23F multiresistant clone. Similarly, most of the serogroup 6 isolates were members of the major multiresistant serotype 6B clone, or variants of this clone. Eighteen of the 24 isolates of serogroup 19 were members of a highly penicillin-resistant clone that appears to be a serotype 19F variant of the major Spanish serotype 23F multiresistant clone. Eighteen of the 25 isolates of serotype 14 were members of a previously uncharacterized highly penicillin-resistant clone. Thirteen of the 16 isolates of serogroup 15 were members of a single previously unreported clone of serotype 15F that had moderate levels of resistance to penicillin. Approximately 65% of the multiresistant pneumococci that are currently circulating in Spain were members of the three new clones of serotype 14, 15F and 19F that we describe here, or the previously described serotype 6B and 23F clones. The other 35% of isolates were minor variants of the major clones, unrelated minor clones, and unique isolates, many of which appeared to have arisen by horizontal gene transfer events.

Keywords: penicillin resistance, pneumococci, molecular epidemiology, penicillin-binding proteins, horizontal gene transfer

INTRODUCTION

Penicillin-resistant isolates of *Streptococcus pneumoniae* in Spain were first recorded in the late 1970s (Casal, 1982; Baquero *et al.*, 1991). Their incidence has increased steadily since then and, in recent years, > 40% of all isolates from Spain have MICs of ≥ 0.1 μg benzylpenicillin ml^{-1} (Fenoll *et al.*, 1991; Liñares *et al.*, 1992; Bouza & Muñoz, 1995). About 35% of these pneumococci exhibit high-level resistance to penicillin (MICs >

1 μg ml^{-1}). Multiply antibiotic-resistant pneumococci are also common in Spain; about 67% of penicillin-resistant isolates are resistant to two or more unrelated antibiotics, with resistance to penicillin, tetracycline and chloramphenicol being the most frequently encountered profile (Fenoll *et al.*, 1991). About 16% of all pneumococci recovered in Spanish hospitals are resistant to these three antibiotics (Bouza & Muñoz, 1995). The incidence of antibiotic-resistant pneumococci in Spain remains among the highest in the world (Baquero, 1995), and the widespread dissemination of multiply antibiotic-resistant pneumococci (Dowson *et al.*, 1994), and difficulties in the management of some serious pneumococcal disease caused by these strains, are a cause of increasing concern (Friedland & McCracken, 1994; Bouza & Muñoz, 1995).

Abbreviations: MLEE, multilocus enzyme electrophoresis; PBP, penicillin-binding protein; REP-PCR, repetitive element primer-PCR; UPGMA, unweighted-pair group method using average linkages.

Table 1. Multiresistant strains of *S. pneumoniae*

Prefixes of isolates refer to the hospitals in which they were recovered: B, Bellvitge, Barcelona; C, Cruces, Bilbao; GA, Gral. de Asturias, Oviedo; GM, Gregorio Marañón, Madrid; MS, Miguel Servet, Zaragoza; NSM, Nostra Senora del Mar, Barcelona; S, Sabadell, Barcelona; SJ, San Jaime, Barcelona; SJD, San Juan de Dios, Barcelona; SO, Severo Ochoa, Madrid; T, Tarrasa, Barcelona; VA, Virgen de la Arrixaca, Murcia; VH, Valle de Hebrón, Barcelona; VR, Virgen del Rocío, Seville. Isolates with the prefix SP are reference strains of previously defined Spanish clones. Antibiotics: Pen, benzylpenicillin; Ery, erythromycin; Cml, chloramphenicol; Tet, tetracycline; MIC of pen is given in $\mu\text{g ml}^{-1}$; R, resistant; S, susceptible. Site of isolation: NK, not known; peri. fluid, pericardial fluid. NS, Non-serogroupable.

Isolate	Isolation		Susceptibility to:				Fingerprint pattern			Serogroup/ type
	Date	Site	Pen	Ery	Cml	Tet	<i>pbp2b</i>	<i>pbp2x</i>	<i>pbp1a</i>	
SP267	1984	NK	2.0	S	R	R	1	1	1	23F
GM4	1988	Eye	2.0	R	R	R	1	1	1	23
GM6	1988	Blood	2.0	S	R	R	1	1	1	23
GM15	1988	Blood	1.0	S	R	R	1	1	1	23
GM28	1988	Eye	2.0	R	R	R	1	1	1	23
GM45	1988	Pharynx	0.5	S	R	R	1	1	1	23F
GM54	1988	Eye	1.0	S	R	R	1	1	1	23F
GM116	1989	Eye	2.0	S	R	R	1	1	1	23
GM142	1989	Blood	2.0	S	R	R	1	1	1	23
GM145	1989	Lung	1.0	S	R	R	1	1	1	23
GM161	1989	Eye	0.5	S	R	R	1	1	1	23
GM163	1989	Blood	2.0	S	R	R	1	1	1	23
SP521	1988	Sputum	2.0	S	R	R	1	1	1	19F
GM70	1990	Blood	2.0	S	R	R	1	1	1	19
GA71	1990	Nose	2.0	S	R	R	1	1	1	19
GM74	1990	Lung	1.0	S	R	R	1	1	1	19
T75	1990	Ear	2.0	S	R	R	1	1	1	19
T77	1994	Nose	2.0	S	R	R	1	1	1	19
S78	1994	Blood	1.0	S	R	R	1	1	1	19
VA79	1994	Eye	2.0	S	R	R	1	1	1	19
S82	1994	Blood	2.0	S	R	R	1	1	1	19
GM83	1994	Lung	2.0	S	R	R	1	1	1	19
C85	1994	Lung	1.0	S	R	R	1	1	1	19F
SJD86	1994	Blood	1.0	S	R	R	1	1	1	19
GM87	1994	Lung	1.0	S	R	R	1	1	1	19
MS88	1994	Sputum	2.0	S	R	R	1	1	1	19
VA91	1994	Nose	2.0	R	R	R	1	1	1	19
VA92	1994	Eye	1.0	R	R	R	1	1	1	19
VA93	1994	Eye	1.0	R	R	R	1	1	1	19
VA95	1994	Sputum	1.0	R	R	R	1	1	1	19
VA96	1994	Eye	1.0	R	R	R	1	1	1	19
SP665	1988	Ear	2.0	S	S	S	1	1	1	9V
GM5	1992	Eye	1.0	S	R	R	1	1	1	9V
SP681	1986	Eye	1.0	R	R	R	2	2	2	6B
GM17	1988	Abscess	1.0	S	R	R	2	2	2	6B
GM121	1989	Blood	2.0	R	R	R	2	2	2	6
T8	1989	Ear	2.0	S	R	R	28	2	2	6B
T99	1991	Pharynx	0.5	S	R	R	28	2	2	6B
NSM2	1989	Sputum	2.0	S	R	R	21	2	2	6A
MS10	1989	Eye	1.0	R	R	R	21	2	2	6B
GM84	1989	Lung	2.0	R	R	R	21	2	6	6
GM41	1988	Blood	1.0	S	R	R	21	43	6	6
GM133	1989	Wound	0.25	R	R	S	4	41	5	6B
GM134	1989	Wound	0.5	R	R	S	4	41	5	6B
GM135	1989	Lung	0.25	R	R	S	4	41	5	NS

Table 1. (cont.)

Isolate	Isolation		Susceptibility to:				Fingerprint pattern			Serogroup/ type
	Date	Site	Pen	Ery	Cml	Tet	<i>pbp2b</i>	<i>pbp2x</i>	<i>pbp1a</i>	
GM32	1990	Pus	2·0	R	R	R	19	46	25	14
T13	1990	Ear	2·0	S	R	R	19	46	25	14
VH14	1990	Blood	2·0	S	R	R	19	46	25	14
VH17	1990	Blood	1·0	S	R	R	19	46	25	14
VH18	1990	Blood	1·0	S	R	R	19	46	25	14
VH19	1990	Blood	1·0	S	R	R	19	46	25	14
VH20	1990	Blood	1·0	S	R	R	19	46	25	14
VH33	1990	Blood	2·0	R	R	R	19	46	25	14
MS21	1990	Nose	1·0	S	R	R	19	46	25	14
MS22	1990	Lung	2·0	S	R	R	19	46	25	14
T9	1991	Eye	0·5	S	R	R	19	46	25	14
B12	1992	Blood	2·0	R	R	R	19	46	25	14
GM23	1993	Blood	1·0	S	R	R	19	46	25	14
GM24	1993	Blood	1·0	S	R	R	19	46	25	14
B25	1993	Sputum	2·0	S	R	R	19	46	25	14
C30	1994	Eye	1·0	S	R	R	19	46	25	14
C38	1994	Blood	2·0	R	R	R	19	46	25	14
VH40	1994	Blood	1·0	R	R	R	19	46	25	14
SJ37	1994	Blood	1·0	R	R	R	28	46	25	14
B27	1994	Pus	1·0	S	R	R	32	45	25	14
B28	1994	Blood	1·0	S	R	R	33	50	25	14
SO34	1990	Ear	1·0	R	R	R	34	51	25	14
GM57	1988	Blood	0·5	R	R	S	21	1	28	14
GM124	1989	Lung	2·0	S	R	S	21	1	28	14
NSM41	1988	Sputum	1·0	S	R	R	4	12	27	15F
GM44	1989	Pus	0·25	S	R	R	4	12	27	15F
GM49	1989	Lung	0·25	S	R	R	4	12	27	15
B67	1989	NK	0·5	R	R	R	4	12	27	15
NSM43	1989	Pus	0·5	S	R	R	4	12	27	15
GM47	1989	Lung	0·25	S	R	R	4	12	27	15
GM48	1989	Lung	0·25	S	R	R	4	12	27	15
MS50	1990	Lung	0·25	S	R	R	4	12	27	15
GA53	1991	Peri. fluid	0·25	S	R	R	4	12	27	15
C60	1993	Pus	0·25	S	R	R	4	12	27	15
B62	1991	Sputum	0·25	R	R	R	4	12	27	15
C68	1993	Eye	0·25	R	R	R	4	12	27	15
C69	1994	Eye	0·25	R	R	R	4	12	27	15
T58	1993	Peri. fluid	0·25	S	R	R	4	12	26	15A
VH97	1989	Peri. fluid	0·25	S	R	R	4	12	26	21
GM50	1988	Blood	0·5	S	R	R	35	26	5	19
GM79	1989	Eye	2·0	R	R	R	35	26	5	19F
GM99	1989	Blood	0·25	S	R	R	35	26	5	19
GM130	1989	Blood	0·25	R	R	R	35	26	5	19
GM169	1989	Eye	0·25	S	R	R	35	26	5	19
GM172	1989	Blood	0·25	S	R	S	26	26	5	19F
R6	1930s	NK	0·016	S	S	S	16	3	3	NS
UK10	1980s	NK	0·008	S	S	S	16	3	3	19
6306	1940s	NK	0·008	S	S	S	16	3	3	6
6314	1940s	NK	0·008	S	S	S	16	3	3	14

Resistance to β -lactam antibiotics has emerged in pneumococci by the development of altered forms of the high-molecular-mass penicillin-binding proteins (PBPs) that

have decreased affinity for the antibiotic (Hakenbeck *et al.*, 1980; Zighelboim & Tomasz, 1980; Dowson *et al.*, 1994; Coffey *et al.*, 1995b). High-level penicillin resistance is due

to alterations of PBP1a, PBP2x and PBP2b (Barcus *et al.*, 1995), whereas high-level resistance to third generation cephalosporins involves alterations of only PBP1a and PBP2x (Coffey *et al.*, 1995a). Low affinity PBPs have emerged by inter-species recombinational events (Dowson *et al.*, 1989; Laible *et al.*, 1991; Martin *et al.*, 1992a; Coffey *et al.*, 1995b). As a consequence, the *pbp* genes of β -lactam-resistant pneumococci have a mosaic structure, consisting of regions that are similar to those in susceptible pneumococci and regions that are 10–25% diverged in nucleotide sequence.

Previous studies have identified two major clones of multiresistant pneumococci in Spain (Muñoz *et al.*, 1991, 1992; Sibold *et al.*, 1992; Soares *et al.*, 1993; Dowson *et al.*, 1994). The prevalent serotype 23F clone has been identified in Spain since the early 1980s and is resistant to penicillin, chloramphenicol and tetracycline (and sometimes erythromycin). This successful clone has been encountered in several other European countries, including the UK, France and Portugal, and has spread intercontinentally to the USA, Mexico and South Africa (Muñoz *et al.*, 1991; Martin *et al.*, 1992; McDougal *et al.*, 1992; Sibold *et al.*, 1992; Dowson *et al.*, 1994; Klugman *et al.*, 1994; Vaz Pato *et al.*, 1995). In some of these countries (e.g. the UK and the USA) the imported Spanish serotype 23F clone has become the most commonly encountered multiresistant pneumococcus. The prevalent multi-resistant serotype 6B clone has also been recovered in Spain for over a decade, and has recently caused outbreaks of pneumococcal disease in Iceland (Muñoz *et al.*, 1992; Soares *et al.*, 1993).

Most studies on penicillin-resistant pneumococci from Spain have concentrated on the description and spread of the prevalent multiresistant serotype 6B and 23F clones (Muñoz *et al.*, 1991, 1992; Soares *et al.*, 1993). However, penicillin-resistant and multiresistant pneumococci of many other serotypes or serogroups were recovered in Spain during the 1980s (Fenoll *et al.*, 1991), and during the 1990s resistant isolates of some of these serogroups have emerged as important causes of pneumococcal disease. For example, in contrast to the 1980s, in which multi-resistance in Spanish pneumococci was most common in isolates of serogroup 23 and 6, in recent years it is most frequently encountered among isolates of serogroup 14 (A. Fenoll, unpublished results).

In this paper we analyse pneumococci resistant to penicillin, chloramphenicol, and tetracycline isolated in Spain between 1988 and 1994. Our study identifies five major clones of multiresistant pneumococci that currently predominate in this country.

METHODS

Pneumococcal isolates and growth conditions. The pneumococci included in this study were multiresistant isolates from Spain that had MICs ≥ 0.25 μ g benzylpenicillin ml⁻¹, and which were also resistant to chloramphenicol (MIC > 4 μ g ml⁻¹) and tetracycline (MIC > 4 μ g ml⁻¹). A few isolates that were initially considered to be resistant to these three antibiotics, but which

on re-testing were found to be tetracycline-susceptible (MIC < 4 μ g ml⁻¹), are also included.

Those with the GM prefix included multiresistant isolates recovered from patients with pneumococcal disease at Hospital Gregorio Marañón during 1988/89 (García-Leoni *et al.*, 1992) and isolates submitted from this hospital to the Instituto de Salud Carlos III between 1990 and 1994. The isolates with other prefixes were submitted from other Spanish hospitals (for details see Table 1) to the Instituto de Salud Carlos III during 1989–1994.

Pneumococci were grown overnight at 37 °C on brain-heart infusion agar containing 5% (v/v) defibrinated sheep's blood (BHI-B), or catalase (1 unit ml⁻¹), in a 95% (v/v) air/5% (v/v) CO₂ atmosphere. Brain-heart infusion broth plus 0.5% yeast extract (BHI-Y) was used as liquid medium. MICs of antibiotics were determined as described previously (Fenoll *et al.*, 1991). Serogrouping of each isolate was carried out at the Instituto de Salud Carlos III, Majadahonda, Madrid, Spain (Fenoll *et al.*, 1991). Serotyping of selected isolates was carried out at the Central Public Health Laboratories, Colindale, UK.

Multilocus enzyme electrophoresis (MLEE). Pneumococci were grown at 37 °C in 200 ml BHI-Y and were harvested by centrifugation during late-exponential phase. Cell lysates were prepared as described by Sibold *et al.* (1992). Methods for starch gel electrophoresis and assay of enzymes are as described by Selander *et al.* (1986). The enzymes analysed are listed in the footnote to Table 2. Relatedness between isolates was estimated from the MLEE data by the unweighted-pair group method using average linkages (UPGMA), using the programs ETDIRV and ETCLUS which were kindly provided by Dr Tom Whittam.

Fingerprinting and sequencing of *pbp* genes. The *pbp1a*, *pbp2b* and *pbp2x* genes were amplified from chromosomal DNA by PCR using the oligonucleotide primers and conditions used previously (Coffey *et al.*, 1991). The amplified *pbp* gene fragments were digested with frequent-cutting restriction endonucleases (Coffey *et al.*, 1991), and the cohesive ends of the resulting DNA fragments were filled-in with [α -³²P]dNTP, fractionated on non-denaturing polyacrylamide gels, and detected by autoradiography (Coffey *et al.*, 1991).

For each *pbp* gene, isolates with identical (or very similar) fingerprints were identified by comparing the digitized fingerprint patterns obtained from the autoradiographs with all of the other patterns using a Summasketch II Plus digitizer tablet (Summagraphics) and MolMatch software (Ultra-Violet Products). This software computes the sizes of each of the DNA fragments from the *pbp* gene by comparing their mobility to those of reference size markers (pBR322 digested with *Hpa*II). Fragment sizes from each *pbp* gene fingerprint are then compared with the fragment sizes obtained from the fingerprint patterns in a database that contains the *pbp* gene fingerprints of all previously analysed isolates, using user-defined criteria of fragment identity. In this work fragments were considered to be identical if their sizes differed by $\leq 5\%$, since large-format high-resolution polyacrylamide gels were used. The program lists, in descending order of similarity, the isolates in the database that have the highest numbers of fragments in common with the test *pbp* gene fingerprint. Isolates that appeared to have the same, or similar, *pbp* fingerprints were then re-analysed by running the samples in adjacent slots on a fingerprint gel.

Fingerprinting cannot distinguish between all mosaic *pbp* genes, and partial nucleotide sequencing was used in those cases where the same *pbp* genes appeared to be present in distantly related clones (Kell *et al.*, 1993). In some cases alleles that looked to be

identical by fingerprinting were distinguished by sequencing. For example, the *pbp1a* genes from the major Spanish serotype 6B and 23F multiresistant clones are not distinguished by the restriction enzymes we use for fingerprinting (Martin *et al.*, 1992b). They were assigned as allele 1 (serotype 23F-like), or allele 2 (serotype 6B-like), by sequencing codons 605–742 where these alleles differ considerably in sequence. Similarly, the *pbp2b* allele of the minor serotype 19F clone (allele 35) appeared to be identical to allele 1 found in the major serotype 23F multiresistant clone, but sequencing of codons 274–356 and 600–679 showed they were distinct. Direct sequencing of regions of the PCR-amplified *pbp* genes was carried out as described previously (Kell *et al.*, 1993).

Repetitive element PCR. PCR using repetitive element primers (REP-PCR) was performed as described by Versalovic *et al.* (1993).

RESULTS

Classification of Spanish multiresistant pneumococci into fingerprint groups

The major Spanish multiresistant serotype 6B and 23F clones have been well documented (Muñoz *et al.*, 1991, 1992; Martin *et al.*, 1992; Sibold *et al.*, 1992; Soares *et al.*, 1993; Dowson *et al.*, 1994; Klugman *et al.*, 1994). As almost all multiresistant isolates of serogroup 23 from Spain are members of the major 23F clone, we examined isolates of this serogroup obtained from Hospital Gregorio Marañón, but not those submitted to the Instituto de Salud Carlos III. For the same reason we only studied a few of the serogroup 6 isolates from the Instituto de Salud Carlos III. The submitting hospital, the year of isolation, and other properties of the pneumococcal isolates are shown in Table 1.

High-resolution fingerprints of the *pbp1a*, *pbp2b* and *pbp2x* genes from each of the multiresistant pneumococci were obtained. For each *pbp* gene, the fingerprints of the multiresistant isolates were compared and those that appeared to possess indistinguishable mosaic forms of each gene were assigned to a fingerprint group (Kell *et al.*, 1993). In most cases, these groups consisted of isolates that were of the same serogroup or serotype. However, there were exceptions (e.g. isolates NSM2 and MS10, and isolates T58 and VH97), and in these cases the serogroups or serotypes were re-checked. As expected, the *pbp1a*, *pbp2b* and *pbp2x* genes of each of the multiresistant pneumococci gave fingerprints that were different from those of the penicillin-susceptible isolate R6 (Table 1; fingerprint group 16-3-3). Other pneumococci from the pre-antibiotic era (e.g. isolates 6306 and 6314), or recent clinical isolates that are truly penicillin-susceptible (i.e. those that have the same MICs as isolates from the pre-antibiotic era), also had the fingerprint pattern 16-3-3 (e.g. isolate UK10).

Multiresistant pneumococci of serotypes 23F, 19F and 9V within fingerprint group 1-1-1

Thirty of the 95 isolates were assigned to fingerprint group 1-1-1 which defines the *pbp* genes of the major multiresistant Spanish serotype 23F clone. All of these

Fig. 1. Relatedness of multiresistant pneumococci. A dendrogram was constructed from a matrix of pairwise differences between the electrophoretic profiles of the pneumococcal isolates using the UPGMA. Null alleles were ignored. The smallest genetic distance shown corresponds to a difference at one enzyme-encoding locus. The serogroups or serotypes of the isolates are shown in parentheses. SP577 and SP264 are not listed in Table 1 but, together with SP264, are previously described members of the major Spanish serotype 23F multiresistant clone (Coffey *et al.*, 1991).

isolates were serogroup 23 and 19, with the exception of isolate GM5 which was serogroup 9.

The 11 isolates of serogroup 23 obtained from Hospital Gregorio Marañón were likely to be members of the Spanish serotype 23F clone as their *pbp* gene fingerprints were identical to those of the reference strain SP267. Two representative isolates (GM45 and GM54) were serotyped and were shown to express capsular type 23F. Isoenzyme analysis demonstrated that isolates GM45 and GM54 were identical in allele profile and they clustered with the reference strains of the Spanish serotype 23F on the dendrogram produced from the electrophoretic data (Fig. 1). Two of the isolates were resistant to erythromycin in addition to penicillin, chloramphenicol and tetracycline.

The 18 fingerprint class 1-1-1 isolates of serogroup 19, which were from eight different hospitals in Spain, were identical by REP-PCR to the reference strain SP521 (Coffey *et al.*, 1991), a previously described serotype 19F variant of the major Spanish multiresistant serotype 23F clone (data not shown). Isolates VA96 and C85 were examined by MLEE, and differed from each other at one of the 16 loci examined. As expected, these isolates clustered with the members of the serotype 23F multiresistant clone on the dendrogram produced from the MLEE data (Fig. 1). Isolate C85 was serotyped and was

Table 2. Electrophoretic variation among multiresistant pneumococci

ET*	Reference isolate	n†	Enzyme‡															
			DIA	GD2	6PG	LAP	G6P	NSP	ADK	IPO	PGI	CAK	LDH	FTK	PLP	LLP	LGP	EST
1	GM5	1	2	1	1	2	1	1	1	1	1	1	1	1	2	1	2	1
2	SP665	4	2	1	1	1	1	1	1	1	1	1	1	2	2	1	2	1
3	VH14	3	2	2	1	1	2	1	1	1	1	1	1	1	2	1	3	1
4	NSM41	2	1	1	1	1	3	1	1	1	1	1	1	2	2	1	2	1
5	GM44	3	1	1	1	2	3	1	1	1	1	1	1	2	2	1	2	1
6	GM45	3	1	1	1	1	3	2	1	1	1	1	1	2	2	1	1	1
7	GM17	6	2	1	1	1	1	1	1	1	1	1	1	2	2	1	2	2
8	GM79	3	2	2	1	1	2	1	1	1	1	1	1	2	2	1	2	1
9	MS10	1	2	2	1	1	1	1	1	1	1	1	1	1	2	1	2	2
10	T58	1	1	1	1	2	3	1	1	1	1	1	1	2	2	1	2	2
11	VH97	1	2	2	1	1	3	1	0	1	1	1	1	1	2	1	2	2
12	SJ37	1	2	3	1	1	2	1	1	1	1	1	1	1	2	1	3	1
13	B27	3	2	1	1	1	3	1	1	1	1	1	1	1	2	1	3	1
14	GM57	1	2	1	1	1	1	1	1	1	1	1	1	2	2	1	3	1
15	267	2	1	1	1	1	2	2	1	1	1	1	1	2	1	1	1	1
16	VA96	1	1	1	1	1	3	2	1	1	1	1	1	2	2	1	2	1
17	GM84	1	2	1	1	1	1	1	1	1	1	1	1	2	2	1	3	2
18	T8	1	2	1	1	1	1	1	1	1	1	1	1	1	2	1	2	2

* Electrophoretic type.

† Number of isolates with that electrophoretic type.

‡ DIA, diaphorase; GD2, glutamate dehydrogenase; 6PG, 6-phosphogluconate dehydrogenase; LAP, leucine aminopeptidase; G6P, glucose-6-phosphate dehydrogenase; NSP, nucleoside phosphorylase; ADK, adenylate kinase; IPO, indophenol oxidase; PGI, phosphoglucose isomerase; CAK, carbamylate kinase; LDH, lactate dehydrogenase; FTK, fructokinase; PLP, phenylalanyl-leucine peptidase; LLP, leucyl-alanine peptidase; LGP, leucyl-glycyl-glycine peptidase; EST, esterase.

shown to express capsular type 19F. Five of the isolates were resistant to erythromycin in addition to penicillin, chloramphenicol and tetracycline.

The single isolate of serogroup 9 in fingerprint group 1-1-1 (isolate GM5) was identical by *pbp* gene fingerprints and REP-PCR to the reference strain SP665, a member of the previously described clone of penicillin-resistant serotype 9V pneumococci recovered in Spain (Coffey *et al.*, 1991). GM5 was shown to express capsular type 9V and differed from the reference strain, SP665, at 2/16 enzymes assayed by MLEE (Table 2). The *pbp1a*, *pbp2b* and *pbp2x* fingerprints characteristic of these isolates (fingerprint group 1-1-1) are shown in Fig. 2(b). The fingerprints of the *pbp1a*, *pbp2b* and *pbp2x* genes of the penicillin-susceptible strain R6 (fingerprint group 16-3-3) are shown in Fig. 2(a).

Multiresistant pneumococci of serogroup 6

The fingerprint group 2-2-2 characterizes the *pbp* genes of the major multiresistant Spanish serotype 6B clone. As expected, we identified two members of this clone in the isolates from Hospital Gregorio Marañón. These serogroup 6 isolates (GM17 and GM121) were identical by MLEE (or differed by a single null allele) to the reference strain of the major serotype 6B clone (SP681). Isolate

GM17 was serotyped and expressed capsular type 6B. The *pbp1a*, *pbp2b* and *pbp2x* fingerprints characteristic of the major serotype 6B clone are shown in Fig. 2(c).

All of the Spanish pneumococci that are classified within fingerprint group 2-2-2 are members of the major serotype 6B clone (Table 1, and unpublished data). However, there were four isolates that possessed the same *pbp1a* and *pbp2x* alleles as the serotype 6B clone, but had different *pbp2b* alleles. Isolates T8 and T99 were both serotype 6B and were recovered two years apart in the same hospital. The other two isolates (NSM 2 and MS10) were recovered in the same year, but in different hospitals, and were distinguished from isolates T8 and T99 as they had a different allele of *pbp2b*. NSM2 and MS10 differed at only two of the 16 enzymes assayed by MLEE, but the former isolate was serotype 6A, whereas the latter was serotype 6B. One further serotype 6 isolate (GM84) possessed the *pbp2x* allele characteristic of the major Spanish serotype 6B clone, but it had the *pbp2b* allele found in isolates NSM2/MS10, and a novel allele of *pbp1a* allele that was found in one further serogroup 6 isolate (GM41). All of these serogroup 6 isolates appeared to be closely related in overall genotype as they clustered together on the dendrogram produced from the MLEE data (Fig. 1).

NSM2/MS10 and T8/T99 appear to be variants of the major serotype 6B clone that have each acquired novel

Fig. 2. Fingerprints of the *pbp1a*, *pbp2b* and *pbp2x* genes of susceptible and multiresistant pneumococci. (a) Digitized *pbp* gene fingerprints from the penicillin-susceptible strain R6 (fingerprint group 16-3-3). The fingerprints from the multiresistant serotype 23F clone (and the major multiresistant serotype 19F clone) are shown in (b) (fingerprint group 1-1-1), and those from the major multiresistant serotype 6B clone in (c) (fingerprint group 2-2-2). In each panel, the digitized patterns of the DNA fragments obtained by digestion of PCR-amplified *pbp2b* with *Sst*I (lane A) and *Hinf*I (lane B), amplified *pbp2x* with *Hinf*I (lane C) and *Dde*I + *Mse*I (lane D), and amplified *pbp1a* with *Hinf*I (lane E) and *Dde*I + *Mse*I (lane F) are shown. pBR322 digested with *Hpa*II (lane m) was used as molecular size markers (Coffey *et al.*, 1991). The sizes of the pBR322 *Hpa*II fragments are 622, 527, 404, 309, 242, 238, 217, 201, 190, 180, 160, 147, 123, 110, 90, 76, 67, 34 and 26 bp.

pbp2b alleles. The *pbp2b* genes of these two variants both differed markedly from that of the major serotype 6B clone. Thus, the *pbp2b* gene of the major serotype 6B clone (allele 2) was cut into eight fragments with *Hinf*I, and only three of these fragments were common to the *pbp2b* genes of isolates NSM2/MS10 (allele 21) or T8/T99 (allele 28).

Two further isolates of serotype 6B, and one non-typeable isolate, were recovered from different patients on different wards in Hospital Gregorio Marañón in June 1989. These isolates (GM133–135) were indistinguishable by MLEE, and appeared to be closely related to the other serogroup 6 isolates, although they had different *pbp* genes and were susceptible to tetracycline.

Identification of a major multiresistant clone of serotype 14 pneumococci

Eighteen of the 24 multiresistant pneumococci of serotype 14 possessed identical alleles of the three *pbp* genes (fingerprint group 19-46-25). All of these 18 isolates gave the same pattern of DNA fragments by REP-PCR (data not shown), and three of the isolates (VH14, GM23 and C38) were examined by MLEE and were indistinguishable. Members of this multiresistant Spanish serotype 14 clone were identified among isolates recovered between 1990 and 1994 from six Spanish hospitals. All of the isolates, except T9 (MIC of 0.5 µg ml⁻¹), had high-level resistance to penicillin (MICs of 1–2 µg ml⁻¹), and five of them, from four different Spanish hospitals, were resistant also to erythromycin. The *pbp1a*, *pbp2b* and *pbp2x*

fingerprints characteristic of this major serotype 14 multiresistant clone are shown in Fig. 3(a).

One further serotype 14 isolate (SJ37) possessed *pbp1a* and *pbp2x* genes that appeared to be the same as those of the serotype 14 clone, but had a markedly different *pbp2b* gene (only 3/8 of the *Hinf*I fragments, and 1/5 of the *Sst*I fragments, from this gene were common to those from the *pbp2b* gene of the major serotype 14 clone). The *pbp2b* gene of isolate SJ37 appeared to be the same as that in two variants of the major serotype 6B clone (allele 28). SJ37 was shown to differ from members of the major serotype 14 clone at only 1/16 enzymes assayed by MLEE.

Three additional serotype 14 isolates (B27, B28 and SO34) possessed the same *pbp1a* allele as the serotype 14 clone, but each had distinct *pbp2b* and *pbp2x* alleles. Two of these isolates (B27 and B28) were recovered in the same hospital in the same year. The third isolate (SO34) was recovered at a separate hospital four years previously and was resistant also to erythromycin. All three isolates were identical by MLEE, except that SO34 differed by one null allele. Cluster analysis suggested that these isolates were also closely related to the members of the major serotype 14 multiresistant clone (Fig. 1).

Two serotype 14 isolates (GM57 and GM124) recovered at the Hospital Gregorio Marañón in 1988 and 1989 were not closely related to the other serotype 14 isolates (Fig. 1) and appeared to have the *pbp2x* gene characteristic of the major multiresistant 23F clone (allele 1), and the *pbp2b* gene found in four of the serogroup 6 isolates (allele 21), combined with a unique *pbp1a* allele. Both isolates were

Fig. 3. Fingerprints of the *pbp1a*, *pbp2b* and *pbp2x* genes of multiresistant pneumococci. The digitized fingerprints of the *pbp* genes amplified from the major multiresistant serotype 14 clone (fingerprint group 19-46-25) are shown in (a), those of the multiresistant serotype 15F clone (fingerprint group 4-12-27) in (b), and those of the minor multiresistant serotype 19F clone (fingerprint group 35-26-5) in (c). In each panel, the digitized patterns of the DNA fragments obtained by digestion of *pbp2b* with *StyI* (lane A) and *HinfI* (lane B), *pbp2x* with *HinfI* (lane C) and *DdeI*+*MseI* (lane D), and *pbp1a* with *HinfI* (lane E) and *DdeI*+*MseI* (lane F) are shown. pBR322 digested with *HpaII* (lane m) was used as molecular size markers.

susceptible to tetracycline, unlike members of the major serotype 14 clone.

Identification of a major multiresistant clone of serogroup 15 pneumococci

There were 14 multiresistant pneumococci of serogroup 15 in the collection. Thirteen of these were in the same fingerprint group (4-12-27). Isolates NSM41, GM44, B67 and C68 were analysed by MLEE and expressed indistinguishable allozymes for each of the 16 enzymes assayed, with the exception of isolate NSM41, which differed at a single locus. Cluster analysis suggested that the genetic background of these isolates was similar to that of the members of the major multiresistant serotype 23F clone and the serotype 19F variants of this clone (Fig. 1). This was supported by REP-PCR which failed to distinguish these isolates from the serotype 23F and 19F isolates. Isolates NSM41 and GM44 were serotyped and were shown to express capsular type 15F.

All of the isolates of this multiresistant serogroup 15F clone, which were recovered from six Spanish hospitals between 1988 and 1994, had intermediate levels of resistance to penicillin (MICs of 0.25–1 µg ml⁻¹). Four isolates, from two different hospitals, were resistant also to erythromycin. The *pbp1a*, *pbp2b* and *pbp2x* fingerprints characteristic of this major serotype 15F clone are shown in Fig. 3(b).

One other isolate of serogroup 15 (T58), appeared to have the same *pbp2b* and *pbp2x* genes as the members of the

serotype 15F clone, but had a distinguishable *pbp1a* gene that possessed one additional *HinfI* site (fingerprint group 4-12-26). T58 appeared by MLEE to be closely related to the other serogroup 15 isolates although it expressed capsular type 15A rather than 15F (Fig. 1). A serogroup 21 isolate (VH97) also appeared to have the identical *pbp1a*, *pbp2x* and *pbp2b* genes as T58, but these isolates were not closely related, differing at 4/16 loci and by one null allele (Fig. 1).

Other multiresistant isolates

There were five isolates of serogroup 19 that appeared to have the same altered *pbp* genes (fingerprint group 35-26-5; Fig. 3c), and one further isolate of the same serogroup (GM172) that differed only in its *pbp2b* gene (fingerprint group 26-26-5). Isolates GM79 and GM172 were serotyped and were shown to express capsular type 19F. These six pneumococci were recovered between June 1988 and October 1989 from six different patients in five wards of Hospital Gregorio Marañón and, excepting GM79, which showed high-level resistance to penicillin, had MICs of 0.25–0.5 µg penicillin ml⁻¹. The high-level penicillin-resistant isolate GM79, and isolate GM130, were resistant to erythromycin in addition to penicillin, chloramphenicol and tetracycline. Three of the isolates, including GM172, were analysed by MLEE and were identical at all 16 loci examined. These multiresistant serotype 19F isolates were, however, not closely related to the serotype 19F variants of the 23F multiresistant clone (Fig. 1). Isolate GM172 was indistinguishable from the other five serotype

19F isolates, except in the fingerprint of the *pbp2b* gene. The *pbp2b* gene of this serotype variant (allele 26) differed from the *pbp2b* gene of the other isolates (allele 35) at 30/240 (12.5%) of the nucleotide sites within the region that was sequenced (codons 274–353).

Nine of the 95 multiresistant pneumococci appeared to be unique isolates that possessed alleles of the *pbp* genes that were not found in any of the other pneumococci. These isolates are not included in Table 1 and were not studied further.

DISCUSSION

Multiple-antibiotic resistance in pneumococci from Spain is currently associated predominantly with isolates of serogroups 14, 23, 6, 19 and 15. All of the serogroup 23 isolates studied here were members of the major Spanish serotype 23F clone. Similarly, most of the serogroup 6 isolates studied here were members of the major Spanish serotype 6B clone, or variants of this clone. The main novel finding in this paper is the rise to prominence in Spain of three new major clones of multiresistant pneumococci of serotypes 14, 15F and 19F.

Multiresistant pneumococci of serotype 14 now predominate in Spain, but they have not previously been studied in any detail. Several distinct clones of serotype 14 isolates were identified, but by far the most prevalent were those of fingerprint group 19-46-25. These isolates, which we shall call the major Spanish multiresistant serotype 14 clone, exhibit high-level resistance to penicillin (MICs $1-2 \mu\text{g ml}^{-1}$), chloramphenicol and tetracycline and, in some cases, to erythromycin. The prevalence of this clone in Spain is difficult to ascertain since the isolates included in our study are necessarily a biased sample of the population of multiresistant pneumococci from Spanish hospitals. However, as serotype 14 isolates are currently the most commonly recovered multiresistant pneumococci in Spain (A. Fenoll, unpublished data), and 75% of the isolates of this serotype were members of the major clone, it is likely that this clone is now more prevalent in Spain than the major serotype 23F or 6B clones. The major multiresistant serotype 14 clone is widely disseminated in Spain but it is at present unclear to what extent it has spread outside of Spain.

The multiresistant serogroup 15 pneumococci were almost all members of a single clone of serotype 15F with moderate levels of resistance to penicillin (MICs of $0.25-1 \mu\text{g ml}^{-1}$). This clone has also not been reported previously, but it appears to be widely disseminated within Spain, as it was recovered from six different hospitals.

Multiresistant serogroup 19 isolates have increased in frequency in Spain during the last five years, and two distinct clones of this serogroup were identified in this work. The major clone was the serotype 19F variant of the Spanish 23F clone which has been proposed to have arisen by the replacement of the genes specifying capsular type 23F with those specifying serotype 19F (Coffey *et al.*,

1991). Two members of this variant have been previously reported from Barcelona (Coffey *et al.*, 1991; Sibold *et al.*, 1992), and one from the USA (McDougal *et al.*, 1992). The serotype 19F clone is highly resistant to penicillin (MIC $1-2 \mu\text{g ml}^{-1}$), and now appears to be widely disseminated within Spain, as isolates were recovered from eight of the hospitals that submitted resistant pneumococci to the Instituto de Salud Carlos III. The minor clone of serotype 19F multiresistant pneumococci (fingerprint group 35-26-5) was recovered only from Hospital Gregorio Marañón in Madrid in 1988/89. The six isolates were recovered over a 17 month period, but it is not clear whether this clone is widespread within Spain. The increase in the frequency of multiresistant pneumococci of serogroup 19 in Spain in recent years is undoubtedly due to the increase in frequency of the serotype 19F variant of the major serotype 23F clone.

Serogroup 9 isolates are the most commonly encountered pneumococci in Spain that are resistant only to penicillin (Fenoll *et al.*, 1991). Most of these isolates are members of the major Spanish penicillin-resistant serotype 9V clone (Coffey *et al.*, 1991; Reichmann *et al.*, 1995; Gasc *et al.*, 1995). Isolate GM5 possessed the same *pbp1a*, *pbp2x* and *pbp2b* genes as the penicillin-resistant serotype 9V isolates and appeared to be similar in overall genotype, differing at 2/16 loci. Isolate GM5 may be a member the serotype 9V clone that has gained additional resistance to chloramphenicol and tetracycline.

Pneumococci are naturally transformable and horizontal transfer of mosaic *pbp* genes (Dowson *et al.*, 1989, 1994) has been proposed to make a significant contribution to the spread of penicillin resistance in pneumococci (Coffey *et al.*, 1991). One of the advantages of using *pbp* gene fingerprinting, rather than PBP profiling (Muñoz *et al.*, 1992), is that it allows the horizontal transfer of *pbp* genes to be detected. Several examples of putative horizontal transfer events were detected in the multiresistant pneumococci studied here. One clear example is provided by isolates T58 and VH97, which were not closely related by MLEE, and were of different serotype, but possessed the same mosaic forms of each of the three *pbp* genes. One possible explanation is that the three *pbp* genes have been transferred horizontally between these isolates.

In other cases, there were isolates that were indistinguishable, except for a difference in the fingerprints of one of the three *pbp* genes. For example, the serotype 19F isolate, GM172, was indistinguishable from the other members of the minor serotype 19F clone (e.g. isolates GM79 and GM99) by MLEE, and appeared to have identical *pbp1a* and *pbp2x* genes, but had a distinct *pbp2b* gene. Similarly, isolate SJ37 appeared to be a member of the major serotype 14 clone that possessed a different *pbp2b* allele, and isolates T99 and NSM2 differed from the major serotype 6B clone only in each having distinct *pbp2b* alleles.

These differences in a single *pbp* gene, between isolates that appear to be members of the same clone, could be due to mutational events that introduce or remove a restriction

site within that *pbp* gene. However, in each of the above examples the differences in the *pbp* gene fingerprints would require the gain or loss of multiple restriction sites. The clonal variants are thus much more likely to be due to recombinational events that have replaced part, or all, of one of the *pbp* genes with the corresponding region of the *pbp* gene from another resistant pneumococcus, or from a related streptococcal species. The role of recombination in the generation of these variants was clearly demonstrated in the one example that we examined in more detail. In this case, the difference between the *pbp2b* genes of the serotype 19F clonal variants, GM169 and GM172, are extremely unlikely to have arisen by mutation as the two alleles differed at 30 nucleotide sites within the 240 bp that were sequenced.

Other examples of the putative horizontal transfer of *pbp* genes can be seen by inspection of Table 1, which shows several cases of isolates of the same, or different, serotypes that shared the same mosaic form of one of the three *pbp* genes. One possible example of a serotype change was found. Isolates NSM2 and MS10 were recovered in the same year in different hospitals and appeared to possess identical mosaic forms of each of the three *pbp* genes. They were also shown to be closely related by MLEE, but expressed capsular polysaccharides of type 6A and 6B, respectively. The molecular explanation for the difference in serotype is currently unclear, but similar phenomena have been reported previously (e.g. the serotype 19F variants of the serotype 23F clone), and have been postulated to be due to the exchange of genes specifying capsular type (Coffey *et al.*, 1991; Kell *et al.*, 1993; Barnes *et al.*, 1995).

Horizontal gene transfer may also be invoked to explain the apparent diversification of the members of the major multiresistant serotype 23F clone and the serotype 19F variants of this clone. These isolates differed considerably by MLEE, although all of them clustered within one of the major lineages in the dendrogram of Fig. 1. A similar variation in the allele profiles of members of the serotype 23F clone recovered in the USA was reported by McDougal *et al.* (1992). Since all of these isolates possess the same altered forms of *pbp1a*, *pbp2x* and *pbp2b*, and are resistant to three unrelated classes of antibiotics, the common ancestor of these isolates cannot realistically predate the introduction of antibiotics into medicine. It has been estimated that a mean of about 26 nucleotide substitutions are required to produce an observable difference in the electrophoretic mobility of a house-keeping enzyme on starch gels (Boyd *et al.*, 1994). It thus seems unlikely that the differences at up to 4/20 enzyme-encoding loci among members of the serotype 23F clone (McDougal *et al.*, 1992; T. J. Coffey, M. Daniels & B. G. Spratt, unpublished data) could have originated by mutation in less than 50 years. A more likely explanation is that the rate of recombination in the naturally transformable pneumococcus is relatively high, and that the initial uniformity of clones is dissipated by localized recombinational events that occasionally replace the alleles at loci that are analysed by MLEE with alleles from other pneumococcal lineages (Maynard Smith *et al.*, 1991).

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