Epidemiol. Infect. (1997), **118**, 105–110

Genetic relationship of penicillin resistant *Streptococcus pneumoniae* serotype 19B strains in Japan

R. YOSHIDA[1], Y. HIRAKATA[1], M. KAKU[1], H. TAKEMURA[1], H. TANAKA[1], K. TOMONO[2], H. KOGA[2], S. KOHNO[2] AND S. KAMIHIRA[1]

[1] *Department of Laboratory Medicine and* [2] *Second Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki-City, 852 Japan*

(*Accepted 27 October 1996*)

SUMMARY

Pulsed field gel electrophoresis (PFGE) of the genomic DNA of penicillin resistant serotype 19B *Streptococcus pneumoniae* was carried out. Thirteen strains form the Nagasaki area and 12 strains from other areas in Japan were examined. Twenty-three strains were resistant to erythromycin, tetracycline and trimethoprim/sulfamethoxazole but susceptible to chloramphenicol. Eight strains were resistant to ceftriaxone. All strains were multiply resistant. Five strains isolated from Nagasaki were indistinguishable from each other by using restriction enzymes *Apa* I and *Sma* I. Two strains isolated from other areas were indistinguishable from the above five strains. We could classify 13 Nagasaki strains into 3 groups and the total of 25 Japanese strains into 6 groups. These results suggest that the increasing prevalence of multiply drug resistant *S. pneumoniae* serotyped 19B in Japan is not due to a single clone, but at least one clone has spread widely in Japan.

INTRODUCTION

Streptococcus pneumoniae is a part of the normal microflora of the nasopharynx and also an important pathogen, responsible for pneumonia, bacteraemia, meningitis, otitis media, and other infections [1]. It has been estimated that 150000–570000 cases of pneumococcal pneumonia occur in the United States and that 5% of individuals with pneumococcal pneumonia die each year [2]. Recently, it has also become one of the most frequent bacterial pathogens in patients with AIDS in the developing world [3].

In 1967, the first penicillin resistant *S. pneumoniae* was reported in Australia [4]. Since then, penicillin resistant *S. pneumoniae* has been isolated from all over the world. The increase and the spread of these strains worldwide has become a major therapeutic and epidemiological concern [5, 6]. Specific epidemiological markers used to survey penicillin resistant *S. pneumoniae* infections include serotyping, penicillin-binding protein patterns, multilocus enzyme electrophoresis patterns, and pulsed field gel electrophoresis (PFGE) patterns. Penicillin resistant *S. pneumoniae* belong predominantly to serogroups/serotypes 6, 14, 19 and 23 worldwide. [5]. Recent surveillance in Japan showed most of penicillin resistant *S. pneumoniae* to be serogroup 19 or 23 [7]. We also reported that the major penicillin resistant *S. pneumoniae* in the Nagasaki area belonged to serotype 19B [8]. Molecular studies have shown that some resistant strains, which belong to serotype 6B, 23F and serogroup 9, spread over large distances [9–11]. However, no data on the spread of serotype 19B pneumococci have been reported.

In this study, we examined the genetic relationship between penicillin resistant *S. pneumoniae* serotype 19B isolated in our hospital and those in other areas of Japan. Moreover, we examined antimicrobial susceptibility of these strains to several antibiotics.

https://doi.org/10.1017/S0950268896007273

Table 1. *Properties of clinical isolates of serotype 19B penicillin resistant pneumococci*

Strain	Origin	Year	Status*	Source	MIC (μg/ml)†		Resistance pattern†					
					PCG	CTRX	EM	TC	TS	CP	CLDM	VCM
NAG-1	Nagasaki	1988	IP	Sputum	2	0·25	R	R	R	S	S	S
NAG-2	Nagasaki	1990	OP	Sputum	2	0·5	R	R	R	S	R	S
NAG-3	Nagasaki	1991	OP	Sputum	2	1	R	R	R	S	S	S
NAG-4	Nagasaki	1992	OP	Sputum	2	0·5	R	R	R	S	R	S
NAG-5	Nagasaki	1992	IP	Sputum	2	0·5	R	R	R	S	R	S
NAG-6	Nagasaki	1993	IP	Sputum	2	0·5	R	R	R	S	S	S
NAG-7	Nagasaki	1993	IP	Sputum	2	0·5	R	R	R	S	S	S
NAG-8	Nagasaki	1994	IP	Sputum	2	0·5	R	R	R	S	S	S
NAG-9	Nagasaki	1994	IP	Sputum	2	1	S	R	R	S	S	S
NAG-10	Nagasaki	1994	OP	Sputum	2	0·5	R	R	R	S	S	S
NAG-11	Nagasaki	1994	OP	Sputum	4	0·5	R	R	R	S	S	S
NAG-12	Nagasaki	1994	IP	Sputum	2	0·5	R	R	R	S	S	S
NAG-13	Nagasaki	1994	IP	Sputum	2	0·5	R	R	R	S	S	S
JPN-1	Hiroshima	1994	OP	Pleural effusion	2	1	R	R	R	S	R	S
JPN-2	Yokohama	1994	IP	Blood	2	2	R	R	R	S	S	S
JPN-3	Fukuoka	1995	IP	Sputum	2	0·5	R	R	R	S	S	S
JPN-4	Osaka	1995	OP	Sputum	2	0·5	R	R	R	S	S	S
JPN-5	Nagano	1995	OP	Middle ear fluid	2	0·5	R	R	R	S	S	S
JPN-6	Hamamatsu	1994	IP	Middle ear fluid	2	0·5	R	R	R	S	R	S
JPN-7	Nagoya	1994	OP	Sputum	2	1	R	R	R	S	R	S
JPN-8	Akita	1995	OP	Middle ear fluid	2	1	R	R	R	S	R	S
JPN-9	Miyazaki	1995	OP	Middle ear fluid	2	1	R	R	R	S	R	S
JPN-10	Chiba	1995	IP	Sputum	2	2	R	R	R	S	R	S
JPN-11	Chiba	1995	OP	Sputum	4	0·5	S	R	R	R	S	S
JPN-12	Tokyo	1994	OP	Middle ear fluid	2	0·25	R	R	R	S	R	S

* IP, in-patient; OP, out-patient.

† PCG, penicillin G; CTRX, ceftriaxone; EM, erythromycin; TC, tetracycline; TS, trimethoprime/sulfamethoxazole; CP, chloramphenicol; CLDM, clindamycin; VCM, vancomycin; S, susceptible; R, resistant.

METHODS

Bacterial strains and antimicrobial susceptibility

Of 280 *S. pneumoniae* isolated during a 7-year period (1988–94) in Nagasaki University Hospital, 18 penicillin resistant *S. pneumoniae* (PRSP) with penicillin G (PCG) MICs of $\geqslant 2{\cdot}0$ μg/ml were detected. Thirteen of the 18 PRSP belonged to serotype 19B. These 13 PRSP isolated in our hospital and the other 12 serotype 19B PRSP isolated in other hospitals in Japan in 1994–5 (which were randomly selected and provided by H. Kurokawa, Health Science Institute, Yokohama, Japan) were used in this study.

Identification of strains was based on colony morphology, result of Gram stain, bile solubility and optochin susceptibility [1]. MICs of PCG (Meiji Pharmaceutical Co. Ltd, Japan) and ceftriaxone (CTRX; Nippon Roche K. K., Japan) for the isolates were determined by the National Committee for Clinical Laboratory Standards (NCCLS)-recommended broth microdilution method with cation-adjusted Mueller-Hinton broth supplemented with 3% lysed horse blood as previously described [8, 12]. Serotyping was performed by detection of the quellung reaction with specific antisera from the Statens Seruminstitut (Copenhagen, Denmark) [1].

Additional susceptibility studies were also done for erythromycin (EM), tetracycline (TC), chloramphenicol (CP), trimethoprim/sulfamethoxazole (TS), clindamycin (CLDM) and vancomycin (VCM) by NCCLS-recommended disk diffusion method [12]. Antimicrobial disks were purchased from Becton-Dickinson Microbiology Systems, Cockeysville, MD, USA. The susceptibility standards for each antibiotic were defined according to NCCLS break points [12]. Both intermediate resistant and resistant categories were included as resistant in this study.

Chromosomal analysis by PFGE

Genomic DNA was prepared by the procedure described by Lefèvre and colleagues [13]. The DNA

https://doi.org/10.1017/S0950268896007273

fixed in the agarose blocks was preincubated in *Sma* I restriction buffer (33 mM Tris-acetate, pH 7·9, 10 mM MG-acetate, 0·5 mM dithiothreitol, 66 mM K-acetate and 0·01% bovine serum albumin) or *Apa* I restriction buffer (10 mM Tris-HCl, pH 7·5, 10 mM $MgCl_2$ and 1 mM dithiothreitol) at room temperature for 1 h. The blocks were mixed with 250 U/ml of *Sma* I (Takara Shuzo, Co. Ltd, Ohtsu, Japan) at 30 °C or *Apa* I (Takara Shuzo, Co. Ltd, Ohtsu, Japan) restriction enzyme at 37 °C for 20 h.

Plugs containing restricted DNA were cast in 1·5% agarose gel (Agarose NA, Pharmacia LKB, Sweden) by supporting them against a comb while the liquid agarose was poured and allowed to solidify [14]. After solidification, the gel was prepared in the running buffer, 0·5 × TBE (50 mM Tris, 50 mM boric acid, 0·2 mM EDTA [pH 8·0]). A Gene Navigator System (Pharmacia LKB, Sweden) was used. The running condition for *Apa* I- and *Sma* I-digested plugs was 200 V for 22 h with pulse times 9·0 s, and 200 V for 20 h with pulse times 9·5 s, respectively. Lambda DNA ladder (FMC BioProducts, USA) was used as a molecular size marker.

Thereafter, gels were stained with ethidium bromide and destained in distilled water before being photographed under UV transillumination. To interpret chromosomal DNA restriction patterns produced by PFGE, we used the criteria recommended by Tenover and colleagues [15]. Briefly, each strain was classified as indistinguishable, closely related, possibly related, and different if the number(s) of fragment differences compared with a reference strain were 0, 1–3, 4–6, $\geqslant 7$, respectively. If the fragment patterns were indistinguishable, closely related, or probably related in DNA restriction patterns, they were considered as derivatives from a common ancestor.

RESULTS

Antimicrobial susceptibilities of 25 penicillin resistant *S. pneumoniae*

Most of serotype 19B PRSP in this study were resistant to EM, TC and TS but susceptible to CP (Table 1). NAG-2, NAG-4, NAG-5, JPN-1, JPN-6, JPN-7, JPN-8, JPN-9, JPN-10 and JPN-12 were also resistant to CLDM. Moreover NAG-3, NAG-9, JPN-1, JPN-2, JPN-7, JPN-8, JPN-9 and JPN-10 were also resistant to CTRX (MIC, $\geqslant 1\ \mu$g/ml). All strains were susceptible to VCM. Pneumococci with resistance to at least three different classes of antibiotics were defined as multiply resistant [6]. All strains were multiply resistant in this study.

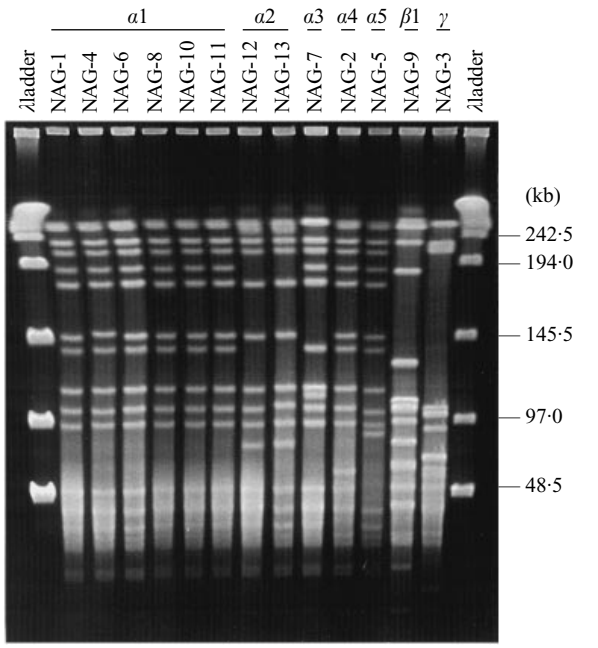

Fig. 1. Pulsed field gel electrophoresis of *Sma* I-digested genomic DNA from 19B PRSP strains isolated in Nagasaki area. The outermost two lanes are λ DNA ladder. Sizes (in kilobases) are indicated on the right. NAG-1, which was first isolated in Nagasaki University Hospital, was used as the reference strain.

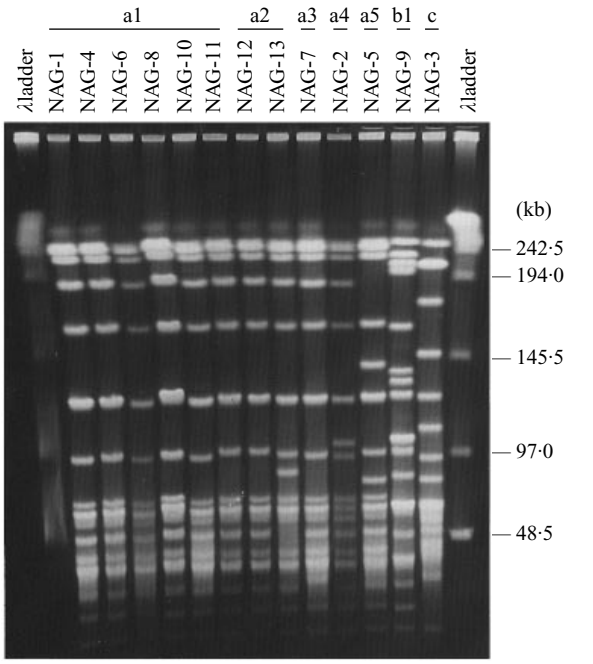

Fig. 2. Pulsed field gel electrophoresis of *Apa* I-digested genomic DNA from 19B PRSP strains isolated in Nagasaki area. The outermost two lanes are λ DNA ladder. Sizes (in kilobases) are indicated on the right. NAG-1 was used as the reference strains.

https://doi.org/10.1017/S0950268896007273

Table 2. *Genetic relationship of 25 serotype 19B penicillin resistant pneumococci by PFGE profiles of* Sma *I and* Apa *I restriction fragments*

Strain	*Sma* I pattern*	*Apa* I pattern*	Grouping by PFGE
NAG-1	α1	a1	Group A
NAG-2	α4(C)	a4(C)	Group A
NAG-3	γ	c	Group C
NAG-4	α1	a3(C)	Group A
NAG-5	α5(C)	a5(C)	Group A
NAG-6	α1	a1	Group A
NAG-7	α3(C)	a2(C)	Group A
NAG-8	α1	a1	Group A
NAG-9	β1	b1	Group B
NAG-10	α1	a1	Group A
NAG-11	α1	a1	Group A
NAG-12	α2(P)	a1	Group A
NAG-13	α2(P)	a1	Group A
JPN-1	α1	a1	Group A
JPN-2	α1	a1	Group A
JPN-3	α1	a8(C)	Group A
JPN-4	α1	a3(C)	Group A
JPN-5	α2(P)	a1	Group A
JPN-6	α6(C)	a6(C)	Group A
JPN-7	α7(C)	a7(C)	Group A
JPN-8	β2(C)	b2(P)	Group B
JPN-9	β3(P)	b3(P)	Group B
JPN-10	δ	d	Group D
JPN-11	ε	e	Group E
JPN-12	φ	f	Group F

* C, closely related; P, possibly related.

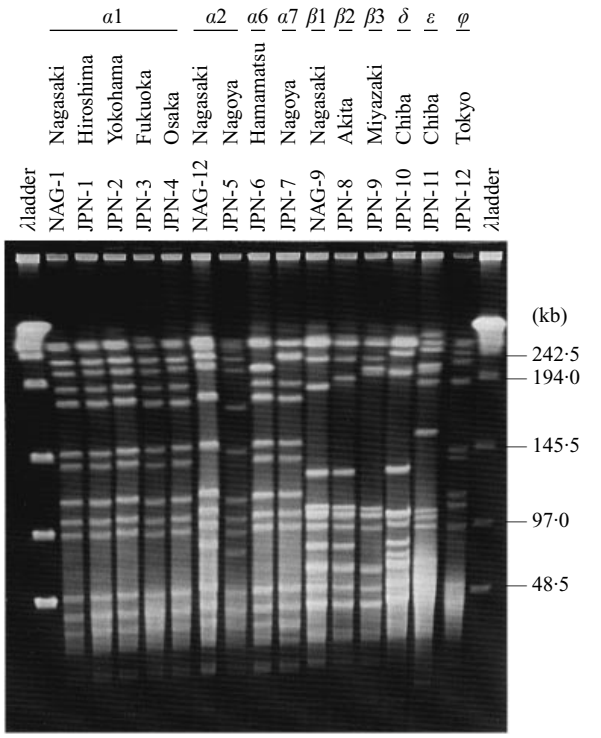

Fig. 3. Comparison between the *Sma* I profiles of Nagasaki strains and those of isolates from other Japanese areas. The *Sma* I profiles of JPN-1, 2, 3, and 4 were indistinguishable from that of NAG-1. The profile of JPN-5 was possibly related to the reference strain (NAG-1) and indistinguishable from that of NAG-12. The profiles of JPN-6 and 7 were closely related. The profiles of JPN-8, 9, 10, 11, 12 were different from that of NAG-1 but JPN-8 and 9 were closely and possibly related to NAG-9.

Chromosomal analysis of pneumococcal DNA by PFGE

To compare these strains by PFGE, we chose strain NAG-1, which was the first clinical 19B PRSP isolated in our hospital, as the reference. The *Sma* I and *Apa* I restriction patterns of five strains, NAG-1, NAG-6, NAG-8, NAG-10, and NAG-11 were indistinguishable from each other and were designated type α1 and type a1 (Figs. 1, 2). The *Sma* I profile of NAG-4 and the *Apa* I profile of NAG-12 and NAG-13 were also identical to the reference. The *Sma* I profiles of strains NAG-12 and NAG-13 were possibly related to the reference profile. The *Sma* I profiles of NAG-2, NAG-5 and NAG-7 were closely related to the reference. NAG-2, NAG-4, NAG-5 and NAG-7 were considered to be closely related strains by the *Apa* I profile. Since not only the *Sma* I profile but also the *Apa* I profile of NAG-9 and NAG-3 differed by at least seven bands comparing to the reference, they were considered to be different strains and were designated β1(b1) and γ (c), respectively. Considering the above results, we could classify 13 PRSP isolated in our hospital into three groups (Group A, B and C) (Table 2).

We compared the PFGE profiles of the above strains to 12 other strains isolated from different areas. *Sma* I profiles of JPN-1, JPN-2, JPN-3 and JPN-4 were indistinguishable from those of NAG-1 (Fig. 3). The *Apa* I profiles of JPN-1, JPN-2 and JPN-5 were the same as NAG-1 (data not shown). The PFGE profiles of JPN-6 and JPN-7 were slightly different from NAG-1 and these strains were considered to be closely related. The *Sma* I and *Apa* I profiles of JPN-8 and JPN-9 were possibly related to NAG-9. The profiles of JPN-10, JPN-11 and JPN-12 were entirely different from those of the previous groups and designated groups D, E, and F, respectively (Table 2).

DISCUSSION

The appearance of penicillin resistant *S. pneumoniae* has already become an important problem all over the world. Several severe infections caused by these

https://doi.org/10.1017/S0950268896007273

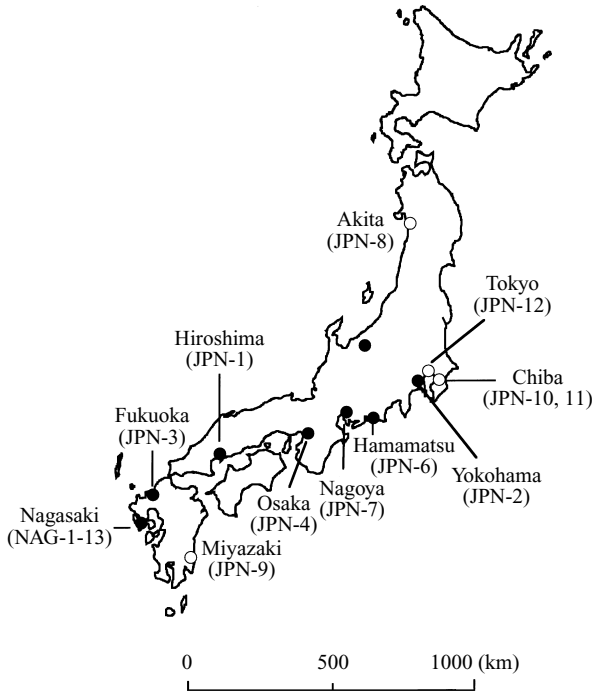

Fig. 4. Map of Japan illustrating the geographic distributions of 25 PRSP. ●, Areas where strain (s) in group A was (were) isolated; ○, ares where strain in group A was not isolated in this study.

resistant strains have been reported [16–18]. In this study, we have investigated the genetic relationships among 19B PRSP in our hospitals and compared them to those isolated in other hospitals in Japan by PFGE. Most 19B PRSP isolated in our hospital belonged to Group A. The PFGE profiles of JPN-1 and JPN-2 isolated far from Nagasaki were indistinguishable from NAG-1 and those of JPN-3, JPN-4, JPN-5, JPN-6 and JPN-7 were genetically related (Fig. 4). This lead us to the conclusion that these strains belonged to the same group. Therefore, it is suggested that Group A strains have spread not only within but also outside the Nagasaki area. The *Sma* I restriction patterns of JPN-8 and JPN-9 were closely and possibly related to NAG-9 (Group B), respectively, and *Apa* I patterns of these two strains were possibly related to NAG-9. However, susceptibility patterns were different from NAG-9. We need to examine other molecular methods to clarify the relatedness of these three strains. The PFGE profiles of other strains were apparently different from Nagasaki strains (Group D–F). These results suggested that the increase of serotype 19B PRSP in Japan was not due to a single clone, but is due to several independent clones and that some strains (Groups A) may have spread widely in Japan.

All Group A strains were resistant to EM, TC and ST. Some strains in this group were also resistant to CLDM. This might mean that their parental clone might be first resistant to EM, TC, TS as like NAG-1, then by selective pressure of antibiotic treatment and finally become resistant to CLDM as well.

Therapy with an extended-spectrum cephalosporin against infections by penicillin resistant *S. pneumoniae* is usually successful. However, *S. pneumoniae* strains resistant to broad-spectrum cephalosporins have recently been reported, together with clinical treatment failure [18–20]. It may be clinically important to survey the spread of PRSP with decreased susceptibility to extended-spectrum cephalosporins. In this study, some strains in group A, which may be a widespread group in Japan, were resistant to CTRX. We need to survey widespread occurrence of clinically important PRSP as like group A thoroughly. Our findings may be of use in the planning aimed at control and possible prevention of the spread of PRSP.

ACKNOWLEDGEMENTS

We thank Fumiaki Iori, Chikako Mochida, Junichi Matsuda, Michiko Nakano and Kazuyuki Sugahara for their technical expertise. We acknowledge the helpful discussions of Peter C. Appelbaum and Michael R. Jacobs.

REFERENCES

1. Musher DM. *Streptococcus pneumoniae*. In: Mandell GL, Douglas RG Jr, Bennett JE, eds. Principles and practice of infectious diseases, 4th ed. New York: Churchill Livingstone Inc, 1995: 1811–26.
2. Centers for Disease Control. Recommendation of the immunization practices advisory committee. Update: pneumococcal polysaccharide vaccine usage – United States. MMWR 1984; **33**: 273–81.
3. Gilks CF. Prophylaxis for HIV-associated infections in the developing world. J Antimicrob Chemother 1993; **31**(Suppl B): 119–28.
4. Hansman D, Bullen MM. A resistant pneumococcus. Lancet 1967; ii: 264–5.
5. Appelbaum PC. Antimicrobial resistance in *Streptococcus pneumoniae*: an overview. Clin Infect Dis. 1992; **15**: 77–83.
6. Klugman KP. Pneumococcal resistance to antibiotics. Clin Microbiol Rev. 1991; **3**: 171–96.
7. Ubukata K, Asahi Y, Okuzumi K, Konno M, The Working Group for Penicillin-Resistant *S. pneumoniae*. Incidence of penicillin-resistant *Streptococcus pneumoniae* in Japan, 1993–1995. J Infect Chemother 1996; **1**: 166–76.

https://doi.org/10.1017/S0950268896007273

8. Yoshida R, Kaku M, Kohno S, et al. Trends in antimicrobial resistance of *Streptococcus pneumoniae* in Japan. Antimicrob Agents Chemother 1994; **39**: 1196–8.
9. Gasc AM, Geslin P, Sicard M. Relatedness of penicillin-resistant *Streptococcus pneumoniae* serogroup 9 strains from France and Spain. Microbiol 1995; **141**: 623–7.
10. Muñoz R, Coffey TJ, Daniels M, et al. Intercontinental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae*. J Infect Dis 1991; **164**: 302–6.
11. Soares S, Kristinsson KG, Musser JM, Tomasz A. Evidence for the introduction of a multiresistant clone of serotype 6B *Streptococcus pneumoniae* from Spain to Iceland in the late 1980s. J Infect Dis 1993; **168**: 158–63.
12. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial susceptibility testing. NCCLS Document M100-S6. Vilanova, PA.: NCCLS; 1995.
13. Lefèvre JC, Faucon G, Sicard AM, Gasc AM. DNA fingerprinting of *Streptococcus pneumoniae* by pulsed-field gel electrophoresis. J Clin Microbiol 1993; **31**: 2724–8.
14. Birren BW, Lai E, Clark SM, Hood L, Simon MI. Optimized conditions for pulsed field gel electrophoretic separations of DNA. Nucl Acids Res. 1988; **16**: 7563–82.
15. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacteria strain typing. J Clin Microbiol 1995; **33**: 2233–9.
16. Appelbaum PC, Bhamjee A, Scragg JN, Hallett AF, Bowen AJ, Cooper RC. *Streptococcus pneumoniae* resistant to penicillin and chloramphenicol. Lancet 1977; ii: 995–7.
17. Shigeno H, Yamasaki T, Nagai H, et al. A case of penicillin resistant pneumococcal pneumonia and penicillin-binding proteins of the clinical isolates. J Jpn Assoc Infect Dis 1992; **66**: 508–14.
18. Bradley JS, Connor JD. Ceftriaxone failure in meningitis caused by *Streptococcus pneumoniae* with reduced susceptibility to beta-lactam antibiotics. Pediatr Infect Dis 1991; **10**: 871–3.
19. John CC. Treatment failure with use of third-generation cephalosporin for penicillin- resistant pneumococcal meningitis: case report and review. Clin Infect Dis 1994; **18**: 188–93.
20. Sloas MM, Barett FF, Chesney PJ, et al. Cephalosporin treatment failure in penicillin- and cephalosporin-resistant *Streptococcus pneumoniae* meningitis. Pediatr Infect Dis 1992; **11**: 662–6.

https://doi.org/10.1017/S0950268896007273