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Molecular Epidemiology of *Streptococcus pneumoniae* with Decreased Susceptibility to Penicillin in a Paris Children's Hospital

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Among pneumococci with decreased susceptibility or pneumococci resistant to penicillin (PRP) isolated at Armand-Trousseau children's hospital, those expressing capsular serotypes 23F, 9V, and 14 were the most frequently isolated. We compared 53 clinical isolates (14 type 9V, 26 type 23F, and 13 type 14) by analysis of chromosomal macrorestriction patterns and DNA restriction patterns of the penicillin-binding protein (PBP) genes *pbp 2b*, *pbp 2x*, and *pbp 1a*. All 9V isolates originated from the same clone. Five 23F clones were distinguished, the largest of which comprised 20 isolates. The main type 14 clone comprised nine isolates; three other type 14 strains were closely related to the 9V clone, probably by horizontal transfer of capsular biosynthesis genes. Most 23F and type 14 isolates shared the same PBP gene restriction patterns as the 9V clone, suggesting horizontal transfer of altered PBP genes.

Penicillin-resistant pneumococci (PRP) are increasingly isolated worldwide, with variations among countries. *Streptococcus pneumoniae* resistance to penicillin is due to alterations of penicillin-binding proteins (PBPs), reducing their affinity for β-lactams. Three PBPs (2b, 2x, and 1a) have been studied by sequencing their genes (*pbp 2b*, *pbp 2x*, and *pbp 1a*) (4, 7, 10). As in other French hospitals, we have noted a marked increase in PRP. In 1993 to 1994, 57% of 363 pneumococci exhibited decreased susceptibility to penicillin (MIC, >0.1 mg/liter), of which 70% had high-level resistance (MIC, >1 mg/liter) and 52% belonged to serotypes 23F, 9V, and 14. Restriction patterns of PBP genes pulsed-field gel electrophoresis (PFGE) of chromosomal DNA, and multilocus enzyme analysis have been used successfully for epidemiological investigations of PRP (9, 11–13).

In this study, we determined PFGE macrorestriction patterns of chromosomal DNA, and DNA restriction patterns of genes *pbp 2b*, *pbp 2x*, and *pbp 1a* to evaluate the genetic relatedness of 53 PRP (14 type 9V, 26 type 23F, and 13 type 14).

All PRP were from children with otitis media, apart from two isolated from children with arthritis: one child with bacteremia and one with meningitis. Eleven of the 14 type 9V isolates had a low level of penicillin resistance (0.125 ≤ MIC ≤ 1 mg/liter), and the other 3 had a high level of resistance (MIC, >1 mg/liter). All of these isolates were also resistant to co-trimoxazole. Twenty-one of the 26 type 23F PRP had a high level of penicillin resistance and were also resistant to other antimicrobial drugs (tetracycline, erythromycin, co-trimoxazole, or chloramphenicol). Eight of the 13 type 14 isolates had a high level of penicillin resistance. Three were also resistant to co-trimoxazole, and the other 10 had multiple resistance. The penicillin-susceptible reference strain R6 and four penicillin-resistant reference strains (types 23F, 6B, 9V, 14, for which penicillin MICs were 0.5, 1, 2, and 4 mg/liter, respectively) were obtained from Centre National de Référence des Pneumocoques.

The PFGE method was adapted from that described by Lefevre et al. (9). *Apa*I macrorestriction fragments were separated on a 1% agarose gel by electrophoresis (CHEF DR III apparatus; Bio-Rad, Ivry, France) at 220 V, with the switch time ramped from 5 to 20 s over an 18-h period at 14°C. PFGE patterns were analyzed with Tenover's categorization (15).

The *pbp 2b*, *pbp 2x*, and *pbp 1a* genes were amplified from chromosomal DNA by PCR with the primers and conditions described by Dowson et al. (2). The primers were CGTGGG ACTATTTATGACCGAAATGG/AATTCCAGCACTGAT GGAAATAAACATATTA for *pbp 2x*, yielding a 2.0-kb fragment (7); GATCCTCTAAATGATTCTCAGGTGG/CAATT AGCTTAGCAATAGGTGTTGG for *2b*, yielding a 1.5-kb fragment (3); and CGGCATTCGATTTGATTCGCTTCT/ C TGAGAAGATGTCTTCTCAGG for *pbp 1a*, yielding a 2.4-kb fragment (1, 10). The 20 PCR cycles consisted of 1 min of denaturation at 95°C, 2 min of annealing at 50°C, and 6 min of extension at 70°C in a thermocycler (Perkin-Elmer, Cetus). Amplified fragments were digested with 5 U of *Hin*fI (Boehringer) and electrophoresed in a 2% high-resolution agarose gel (MetaPhor; TEBU) at 100 V for 4 h.

A single pulsotype (12 fragments, 50 to 350 kb) was observed in all 9V isolates, suggesting a clonal origin (Fig. 1A). Six pulsotypes (A, B, C, D, E, and F) were obtained with the 26 type 23F isolates (Fig. 2A). The principal 23F clone (18 isolates) was mainly pulsotype A: 15 subtypes (A1 to A15) were distinguished, showing that the strains were closely related (15). Among the 13 type 14 isolates, 9 had similar pulsotypes (subtypes A1 to A7), forming the dominant clone. Three others were pulsotype B and were closely related to clone 9V. The last isolate was pulsotype C (Fig. 3A).

The restriction patterns of the *pbp 2b*, *pbp 2x*, and *pbp 1a* genes of all of the penicillin-resistant isolates were different from those of the penicillin-susceptible strain R6. All type 9V

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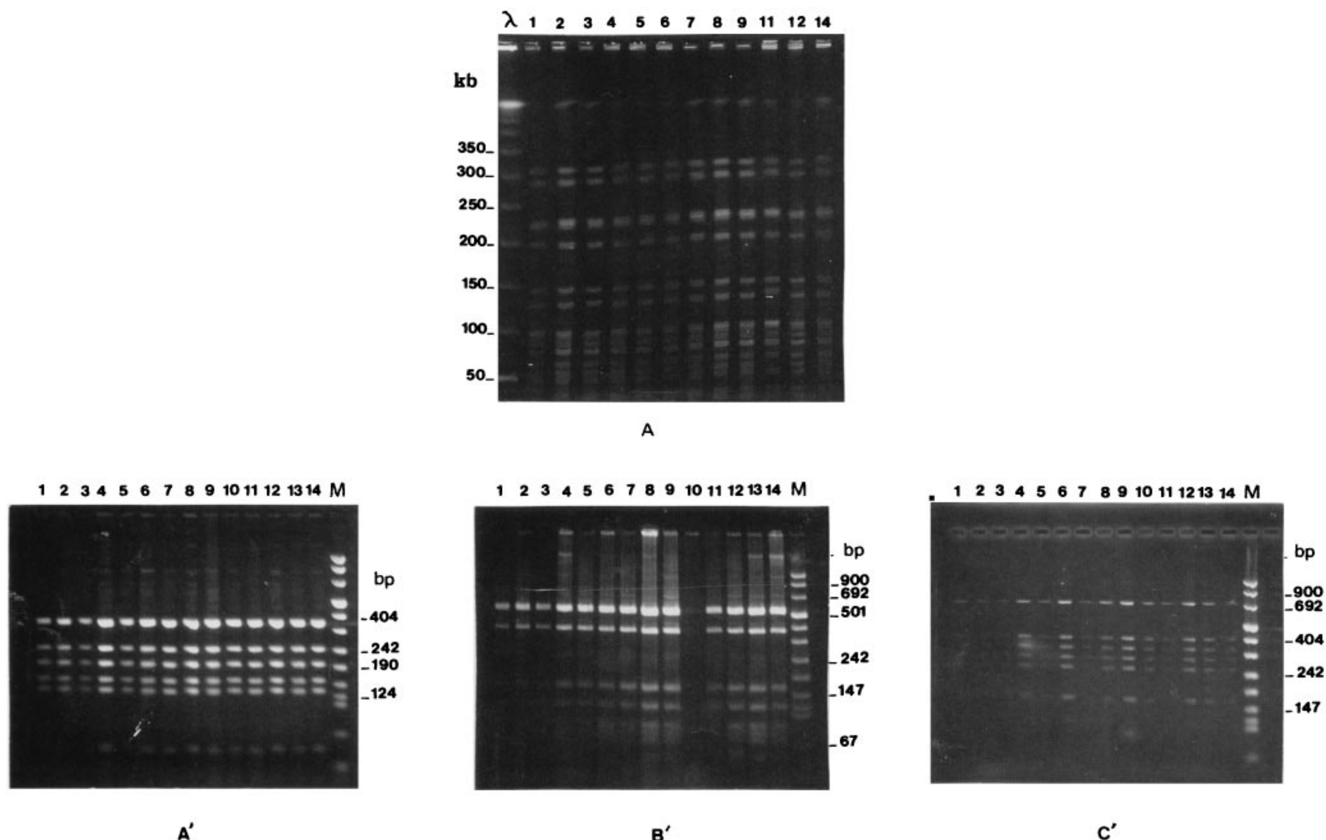

FIG. 1. (A) *Apa*I PFGE patterns of type 9V isolates. All of the isolates have identical patterns. (Patterns of isolates 10 and 13 are not shown but were identical.) (A′, B′, and C′) *Hin*fI restriction patterns of genes *pbp 2b* (A′), *pbp 2x* (B′), and *pbp 1a* (C′) after PCR amplification. The identical profiles confirm the clonal origin of the strains. M, molecular size marker.

isolates, as well as the penicillin-resistant 9V reference, shared the same pattern for each of the three genes, confirming their clonal origin (Figure 1A′, B′, and C′). Twenty-one of the 26 type 23F isolates and 9 of the 13 type 14 isolates shared the same *pbp 2b* gene pattern with clone 9V (Fig. 1A′, 2A′, and 3B). The *pbp 2x* gene pattern obtained with clone 9V was identical to that of 24 of the 26 type 23F isolates and 12 of the 13 type 14 isolates (Fig. 1B′, 2B′, and 3C). The same *pbp 1a* gene pattern was obtained with 22 of the 26 type 23F isolates and 8 of the 9 type 14 isolates and was identical to that of clone 9V (data not shown). These findings show that PFGE is a more discriminatory typing method than either PBP gene analysis or serotyping.

In France, 23F, 9V, 6, 19, and 14 are the most frequently encountered capsular serotypes in pneumococci with reduced susceptibility to penicillin (5, 6). In our institution, the large number of cases of otitis media recruited from the ear, nose, and throat ward explains the predominance of type 23F. Types 9V and 14 came in second and third place, respectively. The frequency of serotype 9V has increased markedly in France since 1989 (1.6% in 1988, 7.8% in 1989, and 11.1% in 1991) (5). The clonal origin of 9V strains, previously suggested by Coffey et al. (1) and Lefèvre et al. (9), was confirmed in our study on the basis of pulsotype and PBP fingerprint identity. The spread of a serotype 23F clone from Spain to the United States was reported in 1991 (12). The origins of type 23F isolates collected at our institution appear heterogeneous, because six distinct clones were clearly identified with a principal clone comprising 18 closely related isolates. Many of the type 23F isolates may have originated from a common ancestor, but multiplication of a single clone is not the only process explaining PRP spread. Nine of the 13 type 14 PRP originated from a common ancestor, and, surprisingly, 3 other isolates were closely related to clone 9V. This may be explained by horizontal transfer and recombination of genes that determine the type 14 capsular polysaccharide from a type 14 strain to a type 9V strain. Coffey et al. in 1991 (1) and Lefevre et al. in 1993 (9) reported the possibility of such transfer in PRP and showed that strains expressing different capsular types could be closely related in genetic terms. Most isolates expressing types 9V, 23F, and 14 harbored identical altered PBP genes. Independent mutation of PBP genes can explain penicillin resistance in several different isolates, but our findings argue against this mechanism and suggest that horizontal transfer of PBP genes between strains is more likely. DNA transfer has been reported between PRP of types 9 and 23 (1, 2) and appears to account for the genetic diversity of PRP isolated in South Africa (14). Furthermore, DNA transfer can occur between viridans group streptococci and *S. pneumoniae* (8, 10).

The acquisition and spread of β-lactam resistance in pneumococci are complex processes, involving clonal diffusion, DNA horizontal transfer, and point mutations. Close contact among children and frequent antibiotic treatment favor the selection and spread of PRP. DNA fingerprinting markers, which provide more precise information than serotyping, are very useful for epidemiological investigations of PRP.

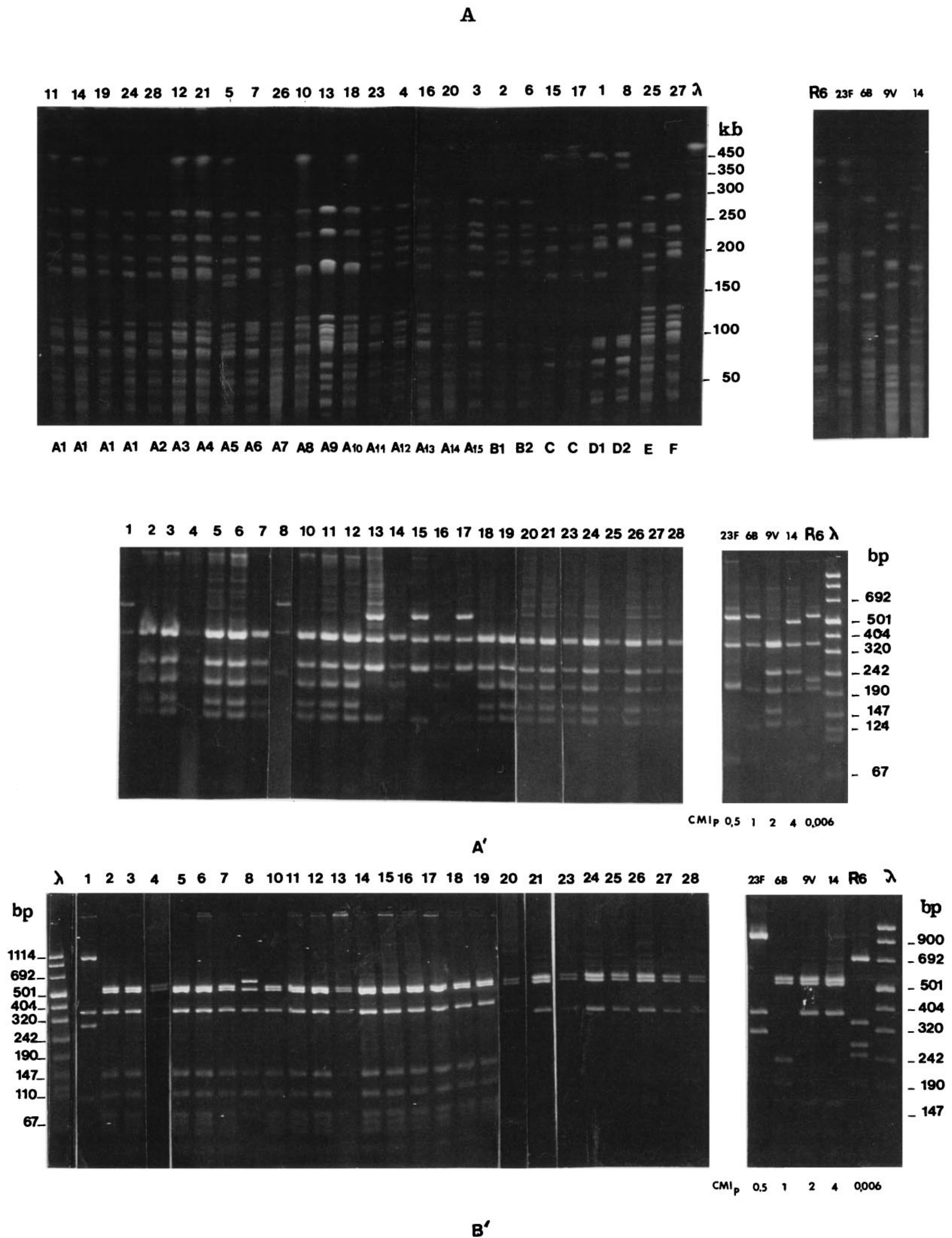

FIG. 2. (A) *Apa*I PFGE patterns of 26 type 23F isolates. The size ladder λ is phage Lambda. R6 is a penicillin-susceptible reference strain. 23F, 6B, 9V, and 14 are penicillin-resistant reference strains. Six pulsotypes (A, B, C, D, E, and F) were produced. Pulsotype A is composed of 15 subtypes (A1 to A15) and is the principal clone, with 18 strains. (A′ and B′) *Hin*fI restriction patterns of the genes *pbp 2b* (A′) and *pbp 2x* (B′). Twenty-one of the 26 type 23F isolates had the same *pbp 2b* gene pattern as clone 9V. The *pbp 2x* gene pattern observed with clone 9V is identical to that of 24 of the 26 type 23F isolates. CMI_p, penicillin MIC.

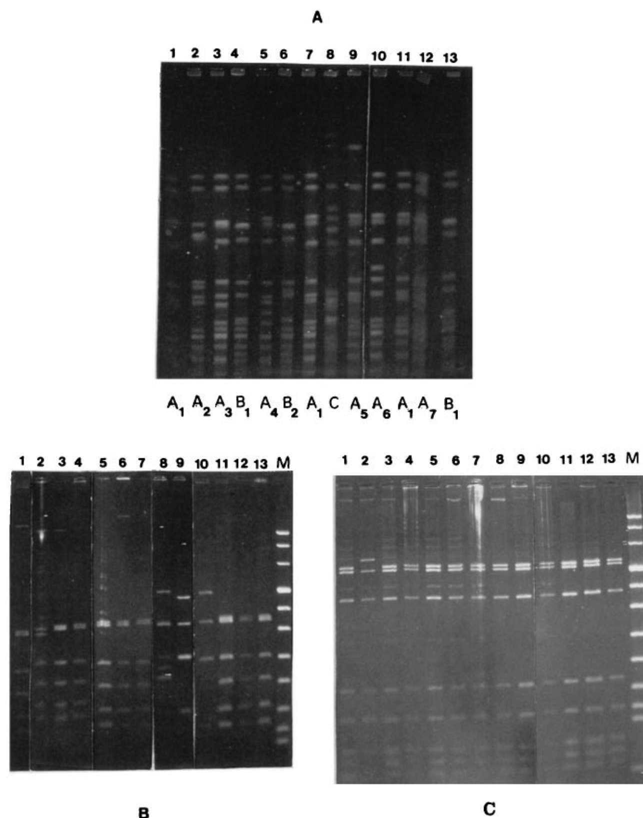

FIG. 3. (A) *Apa*I PFGE patterns of 13 type 14 isolates. Nine strains exhibit pulsotype A (subtypes A1 to A7) and represent the dominant clone, whereas three others exhibit pulsotype B (subtypes B1 and B2) and are closely related to clone 9V. The last isolate is pulsotype C. *Hin*fI restriction patterns of the genes *pbp 2b* (B) and *pbp 2x* (C). Nine of the 13 type 14 isolates had the same *pbp 2b* gene pattern as clone 9V. The *pbp 2x* gene pattern observed with clone 9V is identical to that of 12 of the 13 type 14 isolates. M, molecular size marker.

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