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Molecular Characterization of Penicillin-Resistant *Streptococcus pneumoniae* Isolates Causing Respiratory Disease in the United States

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ABSTRACT

Three hundred twenty-eight (328) penicillin-resistant *Streptococcus pneumoniae* isolates collected in 39 states of the United States between October, 1996, and March, 1997, from (mostly adult) patients with respiratory disease were characterized by microbiological, serological, and molecular fingerprinting techniques, including determination of chromosomal macrorestriction pattern with pulsed-field gel electrophoresis (PFGE) and hybridization with DNA probes specific for various antibiotic resistance genes. The overwhelming majority of the isolates were in five serogroups (23, 6, 19, 9, 14). All isolates had penicillin MIC values of at least 2 μg/ml, but the collection also included isolates with MIC values as high as 16 μg/ml. Virtually all isolates (96.6%) were resistant to trimethoprim/sulfamethoxazole (SXT) and many isolates were also resistant to chloramphenicol (43%), tetracycline (55%), and erythromycin (65%). Resistance to levofloxacin was extremely rare. The molecular fingerprinting methods showed that a surprisingly large proportion (167 out of 328, or 50.9%) of the isolates belonged to two international epidemic clones of *S. pneumoniae:* clone A (127, or 38.7%) with properties indistinguishable from that of the 23F multiresistant "Spanish/USA" clone widely spread in Europe, Asia, Latin America, and South Africa, and clone B (40, or 12.2%) belonging to the "French" serogroup 9/14 clone widely spread in Europe and South America. Virtually all members of clone A were also resistant to chloramphenicol (*cat*+), tetracycline (*tetM*+), and SXT, and about 75% were also resistant to erythromycin (*mefE*+ or *ermB*+). Close to 30% (39 out of 127) of the clone A isolates expressed anomalous serotypes (primarily serotypes 19 and 14, and nontypable) and most likely represented spontaneous capsular transformants. Most of the 40 isolates (35/40) belonging to clone B expressed serotype 9, with five of the isolates expressing serotypes 14 or 19, or were nontypable. All members of this clone were resistant to penicillin and SXT with only occasional isolates showing resistance to macrolides, tetracycline, and chloramphenicol. The combination of microbiological tests and DNA hybridizations also allowed the identification of unusual strains, for instance, isolates that reacted with the *tetM* or *mefE* DNA probes without showing phenotypic antibiotic resistance, an isolate showing phenotypic macrolide resistance without hybridizing with either the *ermB* or *mefE* DNA probes, or isolates that hybridized with both of these DNA probes. In addition to clones A and B, another large portion of the *S. pneumoniae* isolates (112 of 328, or 34.1%) was represented by eight clusters, each with a unique PFGE type. These clusters, together with the clone A and clone B isolates, made up 85% of all the penicillin-resistant isolates identified in this survey in the United States. Both international clones and the unique clusters showed wide geographic dispersal: Clone A was present in 30 of the 39 states and clone B in 18. The data suggest that the major mode of spread of penicillin-resistant pneumococci in the United States is by clonal expansion and that the most significant components (clones A and B) have been imported into the United States from abroad.

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INTRODUCTION

WITHIN LESS THAN FOUR DECADES after their first detection in clinical specimens,[14,17] penicillin-resistant strains of *Streptococcus pneumoniae* have achieved global spread. Several multicenter surveillance studies performed in the United States since the early 1980s have documented a relatively low frequency of penicillin-insensitive *S. pneumoniae* isolates. In a survey performed between the fall of 1987 and the spring of 1988, involving close to 500 *S. pneumoniae* isolates recovered in 15 hospital centers with wide geographic distribution over the United States, the frequency of penicillin-insensitive pneumococci was estimated as 4%. The great majority (3.8%) of these were isolates with penicillin minimal inhibitory concentration (MIC) values between 0.1 to 1.0 μg/ml. Only a single isolate (0.2%) had truly penicillin-resistant MIC value.[16] In a second survey, partly overlapping in time with the previous one (1979–1987), the frequency of penicillin-insensitive pneumococci among 5,469 isolates recovered in 19 hospital centers was estimated as 5%, with the overwhelming majority, again, being in the low-to-intermediate range of penicillin MIC values.[31] More recent multicenter studies documented a dramatic increase both in the total frequency of penicillin-insensitive isolates and also in the proportion of highly resistant strains. A survey performed between July, 1992, and June, 1993, involving close to 800 isolates and 19 participating centers detected increased penicillin MIC value in a total of 23% of the isolates, with 15% being in the low-to-intermediate range, and 7% in the high MIC range.[2] The abrupt increase in resistance was further documented in at least three recent surveys. A 30-center study involving 1,527 isolates collected between November, 1994, and April, 1995, showed, again, that 23% of all isolates had increased penicillin MIC values, the proportion of low-to-intermediate level resistance being about 14% and the high-level resistance 9.5% of the isolates.[8] The two most recent multicenter surveillance studies suggest that the frequency of penicillin-resistant pneumococci has continued to increase in the United States. A 5-month survey during 1997, involving 845 isolates recovered in 27 centers, identified close to 28% of all isolates as being low-to-intermediate resistant and 16% as highly penicillin resistant (a total of 44% of isolates with increased penicillin MIC value).[10] A most recent surveillance project (TRUST, Tracking Resistance in the United States Today), performed between October, 1996, and Spring, 1997, and involving over 9,000 isolates and 434 participating centers distributed over 45 states, detected decreased susceptibility to penicillin among 33.5% of all the isolates, with close to 20% being in the low-to-intermediate range and 13.6% in the high penicillin MIC range.[34]

In parallel with the increased representation of penicillin-resistant strains, there was also a large increase in frequency of resistance to other antimicrobial agents, for instance, resistance to erythromycin increased from 0.3% to over 10% of all isolates between the mid- to early 1980s and the mid-1990s. During the same time period, resistance to trimethoprin/sulfamethoxazole (SXT) increased from about 0.5% to close to 20% of all pneumococcal isolates.

To obtain some insight into the mechanism of spread of antibiotic-resistant *S. pneumoniae* in the United States, we characterized several hundred highly penicillin-resistant isolates collected in the TRUST study, using molecular fingerprinting techniques.

MATERIALS AND METHODS

Bacterial strains

A total of 328 highly penicillin-resistant *S. pneumoniae* isolates (penicillin MIC ≥ 2 μg/ml), collected in the TRUST study,[34] were grown in the laboratory for the preparation of DNA disks.[30] In some of the experiments, an additional 28 penicillin-susceptible or intermediately penicillin-resistant strains from the TRUST study were also characterized. The penicillin-resistant isolates represented a geographically diversified sample: they originated from 39 states and 145 hospital centers (see Table 1). All isolates were from patients with clinically diagnosed respiratory disease, and the majority of these patients were adults. The largest proportion (262/328, or 79.9%) of the isolates came from 10 states (California, Florida, Georgia, Kansas, Kentucky, Nebraska, New York, Ohio, Tennessee, and Texas). The rationale for this preferential selection of strains was to include (i) states bordering Mexico and major ports of entry to the United States (California, Florida, New York, Texas), and (ii) heavily populated states from which a high frequency of penicillin-resistant pneumococci have been reported (California, Florida, Georgia, Kansas, Kentucky, Nebraska, New York, Ohio, Tennessee, and Texas). All 20 isolates showing resistance to levofloxacin were also included in the analysis. Antibiotic susceptibilities were redetermined using the appropriate E-tests in the case of pneumococcal isolates with unusual antibiotypes (for instance, extremely high penicillin MIC values or isolates that showed antibiotic susceptible phenotype in spite of the presence of a resistance gene, as identified by a DNA probe).

Serotypes were determined on the basis of capsular swelling after suspension in antisera (Dako Co., US).

Pulsed-field gel electrophoresis

Preparation of chromosomal DNA, restriction by *Sma*I endonuclease and pulsed-field gel electrophoresis (PFGE) and interpretation of patterns were as described previously.[30,35] A CHEF-DRII apparatus (Bio-Rad, USA) was used for running the gels. Running conditions were 23 hr at 11.3°C at a voltage set of 200V ramped with initial forward time of 1 sec and final forward time of 35 sec. Gels were stained with ethidium bromide and photographed.

Hybridization with DNA probes

Gels to be hybridized were transferred to nylon membranes with the Vacuum Gene System (Pharmacia Biotech, USA). The resulting membranes were probed using the ECL RPN 3001 system (Amersham, UK) according to the manufacturer's recommendations. The molecular weight of the hybridization signals and the corresponding *Sma*I fragments were determined by comparison with molecular weight standards.

Probes

DNA probes for *ermB* and *mefE* genes were obtained through polymerase chain reaction (PCR) using as templates *S. pneu-*

TABLE 1. ORIGIN OF *S. PNEUMONIAE* ISOLATES

State (code)	Number of isolates	Number of isolates represented by				
		International clones			Clusters	Other PFGE types
		A	B	A + B		
California (CA)	54	26	5	31	13	10
Texas (TX)	39	7	9	16	16	7
Georgia (GA)	37	12	1	13	15	9
Florida (FL)	26	15	2	17	8	1
Nebraska (NE)	23	6	2	8	5	10
Ohio (OH)	21	6	2	8	12	1
Tennessee (TN)	19	3	3	6	10	3
New York (NY)	16	12	2	14	0	2
Kentucky (KY)	15	5	0	5	9	1
Kansas (KS)	12	5	2	7	4	1
Alabama (AL)	3	3	0	3	0	0
Colorado (CO)	3	2	0	2	1	0
Connecticut (CT)	3	1	1	2	1	0
Delaware (DE)	3	1	0	1	2	0
Indiana (IN)	3	1	0	1	1	1
Louisiana (LA)	3	0	1	1	2	0
Massachusetts (MA)	3	1	1	2	1	0
Minnesota (MN)	3	1	1	2	1	0
Missouri (MO)	3	2	0	2	1	0
New Jersey (NJ)	3	3	0	3	0	0
Oklahoma (OK)	3	1	0	1	2	0
Pennsylvania (PA)	3	2	0	2	0	1
South Dakota (SD)	3	0	0	0	3	0
West Virginia (WV)	3	1	2	3	0	0
Wisconsin (WI)	3	1	0	1	2	0
Illinois (IL)	2	2	0	2	0	0
Maryland (MD)	2	0	1	1	1	0
Michigan (MI)	2	0	2	2	0	0
Nevada (NEV)	2	1	0	1	0	1
New Hampshire (NH)	2	0	2	2	0	0
Virginia (VA)	2	2	0	2	0	0
Washington (WA)	2	2	0	2	0	0
Arizona (AZ)	1	0	0	0	1	0
Arkansas (ARK)	1	1	0	1	0	0
Maine (ME)	1	0	1	1	0	0
New Mexico (NM)	1	1	0	1	0	0
North Carolina (NC)	1	1	0	1	0	0
North Dakota (ND)	1	0	0	0	1	0
Utah (UT)	1	0	0	0	0	1
Total	328	127	40	167	112	49
(%)	(100)	(38.7)	(12.2)	(50.9)	(34.1)	(15)
Number of PFGE types	38	1	1	2	8	28

moniae strains 02J 1095 (for *ermB*) and 02J 1175 (for *mefE*), and primers GAAAARGTACTCAACCAAATA and AGTAAYGGTACTTAAATTGTTTAC (for *ermB*) and AGTATCATTAATCACTAGTGC and CGTAATAGATGCAATCACAGC (for *mefE*), generated on the basis of sequences published by Sutcliffe et al.[32]

Previously, it has been shown that streptococcal isolates carried chloramphenicol acetyltransferase (CAT) genes belonging to both cat_{pC194} and cat_{pC221} classes.[7,36] To design primers, protein sequences of the CAT determinants belonging to both classes (Gen Bank accession numbers V01277, J01754, K01998, X65462, S50737, X60827, S45036, X02529, and X02872) were aligned and primers were designed to the conserved regions. The pair of primers thus obtained—CATd, TTAGGYTATTGGGATAAGTTA, and CATr, CATGRTAACCATCACAWACAG—was used to amplify a 338-bp fragment internal to the *cat* gene (235–572 bp of the coding region of the *cat* gene of plasmid pC164). To generate the probe template DNA, the chloramphenicol-resistant strain 8249 was used in a PCR reaction where annealing was performed at 47°C for 30 sec and extension at 72°C for 1 min for 30 cycles. Primers used for generating the *tetM* probe were TETMd, TG-

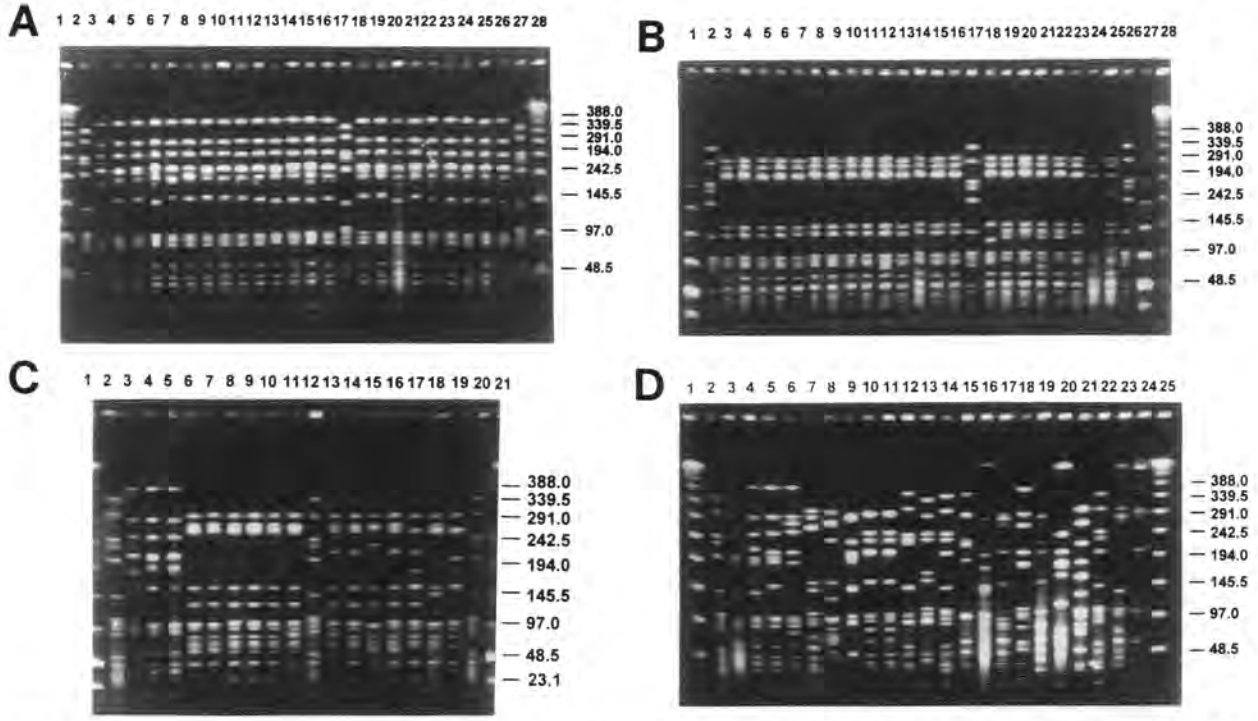

FIG. 1. PFGE patterns of *S. pneumoniae* isolates recovered from various states in the United States. Chromosomal *Sma*I digests were separated by PFGE and photographed in UV light. **(A)** Penicillin-resistant isolates representing international clone A. **(B)** Penicillin-resistant isolates representing international clone B. **(C)** Isolates belonging to cluster 5. **(D)** Isolates resistant to levofloxacin. Positions of *S. pneumoniae* isolates in the gels were as follows. **(A)** Lanes 1 and 28, low molecular weight (LMW) ladder; lanes 2, 17, and 27, laboratory strain R6 used as molecular weight standards; lanes 3–6, representatives of international clone A from Cleveland, Ohio (lane 3), and from South Korrea (lane 4), Mexico (lane 5), and Colombia (lane 6); lanes 22–26, serotype 19 isolates of clone A; lanes 7–16, strains GA38, TX16, AL4, TN3, ARK3, FL4, CA2, NY11, KS4, and VA1; lanes 18–26, strains NE8, OH10, NM1, IL2, KY6, MO2, CO1, NEV1, OK3. **(B)** Lanes 1 and 27, LMW ladder; lane 28, lambda DNA ladder; lanes 2, 17, and 26, laboratory strain R6 used as molecular weight standards; lanes 3–5, representatives of international clone B from outside the United States: Mexico (lane 3), Colombia (lane 4), and Uruguay (lane 5); lanes 6–16 and 18–25, strains CA24, CA52, TX9, TX14, FL17, GA13, OH20, TN16, NY8, NE4, ME1, KS9, NH2, M14, MN1, MD3, WV2, LA1, NE16. **(C)** Lanes 1 and 21, LMW ladder; lanes 2, 12, and 20, laboratory strain R6; lanes 3 and 4, reference strains for international clone A from Cleveland, Ohio (Clev-2) and South Korea (Korea-2); lane 5, GA38, U.S. clone A; lanes 6–11 and 13–19, cluster 5 isolates CA45, CA8, KS11, KY15, KY4, FL5, FL15, GA32, WA2, MA3, FL16, SD4, and KY10. WA2 (lane 15) is susceptible to penicillin. **(D)** Lanes 1 and 25, Lambda ladder; lanes 2, 14, and 22, laboratory strain R6 used as a molecular weight standard; lanes 3–13, 15–21 and 23, 24, PFGE patterns of the 20 levofloxacin-resistant isolates identified in the TRUST Surveillance Project.[34] Lanes 3–6, levofloxacin-resistant isolates from four states in the United States (GA4, PA1, TN2, and CA29) which belong to international clone A; lane 7, a levofloxacin-resistant isolate (WV2) with PFGE type of international clone B; lanes 8–13, WA2, AL2, NY1, NY2, WV1, WA1; lanes 15–21 and 23–24, KY2, TX44, NC1, CA55, TX43, PA2, GA17, AL1, and KY3.

GAATTGATTTATCAACGG, and TETMr, TTCCAACCATACAATCCTTG, which were designed to amplify a 1,080-bp region internal to the *tetM*[20,22] gene and corresponding to nucleotides 529–1,608 of Gen Bank accession number X52632. Preparation of the template and the strain used were as for preparation of the CAT probe. PCR was performed with annealing at 50°C for 30 sec, followed by extension at 72°C for 1 min for 30 cycles.

RESULTS

Characterization of penicillin-resistant S. pneumoniae *by serotyping and PFGE*

Analysis of the 328 penicillin-resistant isolates showed that the largest fraction (50.9%) of the resistant isolates exhibited the two unique PFGE patterns typical of two internationally spread epidemic clones of penicillin-resistant *S. pneumoniae:* The first one, most frequently referred to in the literature as the "Spanish/USA 23F clone,"[22] and arbitrarily referred to here as clone A, was represented by 127 of the 328 isolates (38.7%) (Table 1). The second clone, most frequently referred to in the literature as the "French serotype 9/14 clone,"[13,18] and arbitrarily referred to here as clone B, was represented by 40 isolates (12.2%). Figure 1A shows the PFGE pattern of clone A isolates from 19 different states in the United States. Also included, for comparison, are isolates representing the same clonal type from South Korea (lane 4), Mexico (lane 5), and Colombia (lane 6). Figure 1B shows the PFGE pattern of clone B isolates from 16 different states in the United States, with isolates from Mexico (lane 3), Colombia (lane 4), and Uruguay (lane 5) included for comparison.

In addition to the two international clones, another large portion of the penicillin-resistant *S. pneumoniae* isolates from the United States were represented by eight clusters of strains, each with a unique PFGE type. A cluster was defined as a PFGE pattern shared by at least five *S. pneumoniae* isolates. As many as 112 of the 328 penicillin-resistant isolates (34.1%) belonged to one of these eight clusters. The two international clones, together with the clusters, made up the overwhelming majority (85%) of all penicillin-resistant isolates. Thus, the great majority of the penicillin-resistant strains (279/328) belonged to a relatively few (total of 10) distinct PFGE types. Only 49 out of the 328 resistant strains (15%) were represented by single or up to four independent isolates, and among these 49 strains as many as 28 distinct PFGE types were identified (see Table 1).

Figures 2–5 document the PFGE and DNA hybridization patterns of the U.S. clusters.

Figure 2A shows the macrorestriction pattern of the largest of the U.S. clusters, cluster 18, represented by 31 isolates in this collection. Thirty isolates belonging to this cluster expressed low-level erythromycin resistance (*mefE* probe positive) (Fig. 2B). Interestingly, although none of the 31 strains showed phenotypic tetracycline resistance, as many as 29 out of the 31 isolates gave a positive hybridization signal with the *tetM* DNA probe (see Fig. 2C).

Figure 3A shows the PFGE patterns of isolates belonging to cluster 3. All cluster 3 isolates expressed erythromycin resistance and all isolates hybridized with the *mefE* DNA probe (see Fig. 3B). Most of these isolates (see lanes 3–12) expressed only low-level erythromycin resistance. Interestingly, another group of the cluster 3 isolates (see lanes 14 through 20) hybridized not only with *mefE* but with the *ermB* probe as well (see Fig. 3D), and these isolates expressed high-level resistance to erythromycin. All isolates belonging to this cluster were resistant to tetracycline (*tetM* probe positive) (see Fig. 3C).

Figure 4A illustrates the PFGE patterns of clusters 8 (lanes 2–6), cluster 22 (lanes 8–15), and cluster 16 (lanes 17–22). All five cluster 8 isolates were susceptible to penicillin as well as tetracycline, chloramphenicol, and SXT, were only resistant to low-level erythromycin, and hybridized with the *mefE* DNA probe (Fig. 4B). All cluster 22 isolates were resistant to penicillin (except a single isolate that was susceptible to penicillin). The majority of cluster 22 isolates was also resistant to low-level erythromycin (*mefE* probe positive) and SXT. The seven isolates belonging to cluster 16 were, in addition to penicillin, also resistant to tetracycline (*tetM* probe positive), high-level erythromycin (*ermB* probe positive), and SXT (see Fig. 4C).

Figure 5A shows the PFGE pattern of isolates belonging to cluster 9 (lanes 3–13) and cluster 6 (lanes 15–25). Most of the cluster 9 isolates expressed low-level erythromycin resistance (*mefE* probe positive) and were all resistant to SXT (Fig. 5B). Most of the cluster 6 isolates were resistant to high-level erythromycin (*ermB* probe positive), tetracycline (*tetM* probe positive), and chloramphenicol (*cat* probe positive) (Figs. 5C and 5D). All isolates were also resistant to SXT.

Table 2 summarizes data on the resistance phenotypes and genotypes of the *S. pneumoniae* isolates.

Table 3 documents the distribution of serotypes among the different pneumococcal clones. Out of the 127 strains belonging to international clone A, 88 expressed serotype 23, 37 serotype 19, and a single strain each belonged to either serotype

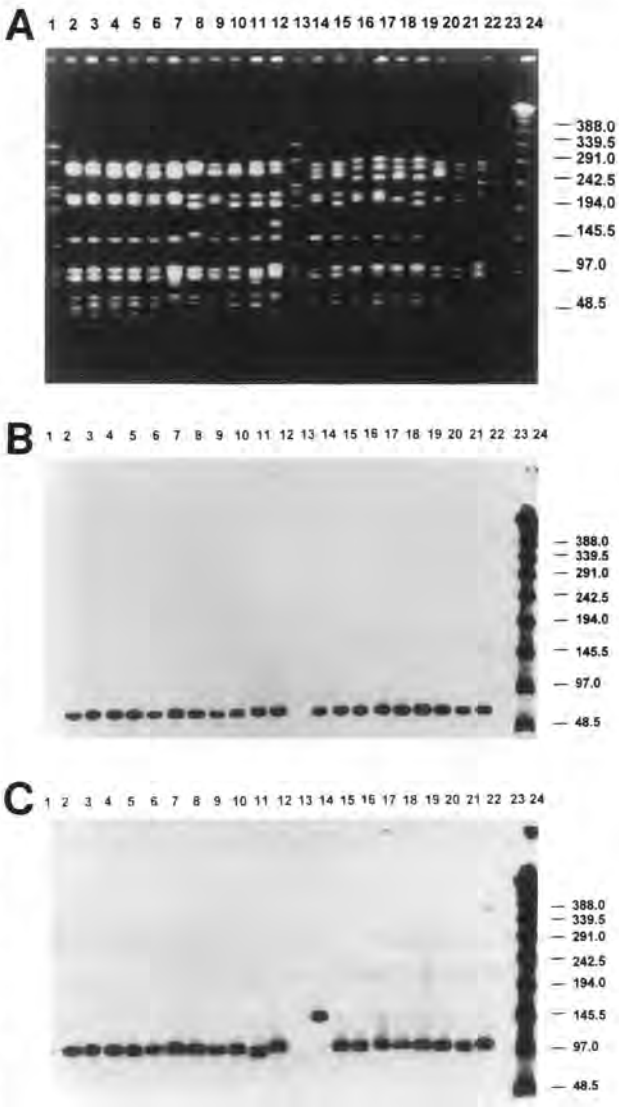

FIG. 2. PFGE and DNA hybridization patterns of penicillin-resistant *S. pneumoniae* isolates from the United States belonging to cluster 18. **(A)** *Sma*I digests of chromosomal DNAs prepared from the strains were separated by PFGE, as described in the Materials and Methods section. DNA fragments were transferred to nylon membranes for Southern hybridization with DNA probes for *mefE* **(B)** and *tetM* **(C)**. Lane 24, Lambda ladder; lanes 1, 13, and 23, laboratory control strain R6 used as molecular weight standard; lanes 2–12 and 14–22, strains TX11, TX22, TX36, TX34, KS12, OK2, TX19, TN10, MD2, SD5, MN2, KY7, TX31, TX37, TX6, OK1, MO1, LA3, KY14, and FL14.

14 or was nontypable. Among the 40 members of international clone B, most (35) expressed serotype 9, one strain belonged to serotype 19, 2 to serotype 14, and two isolates were nontypable. Of the eight distinct U.S. clusters, each had a typical majority serotype. In the case of cluster 18, this was serotype 6, whereas the majority of isolates belonging to clusters 3, 5, 6, and 9 expressed serotypes 19, 23, 6, and 6, respectively. Most strains belonging to clusters 22, 16, and 10 expressed serotypes

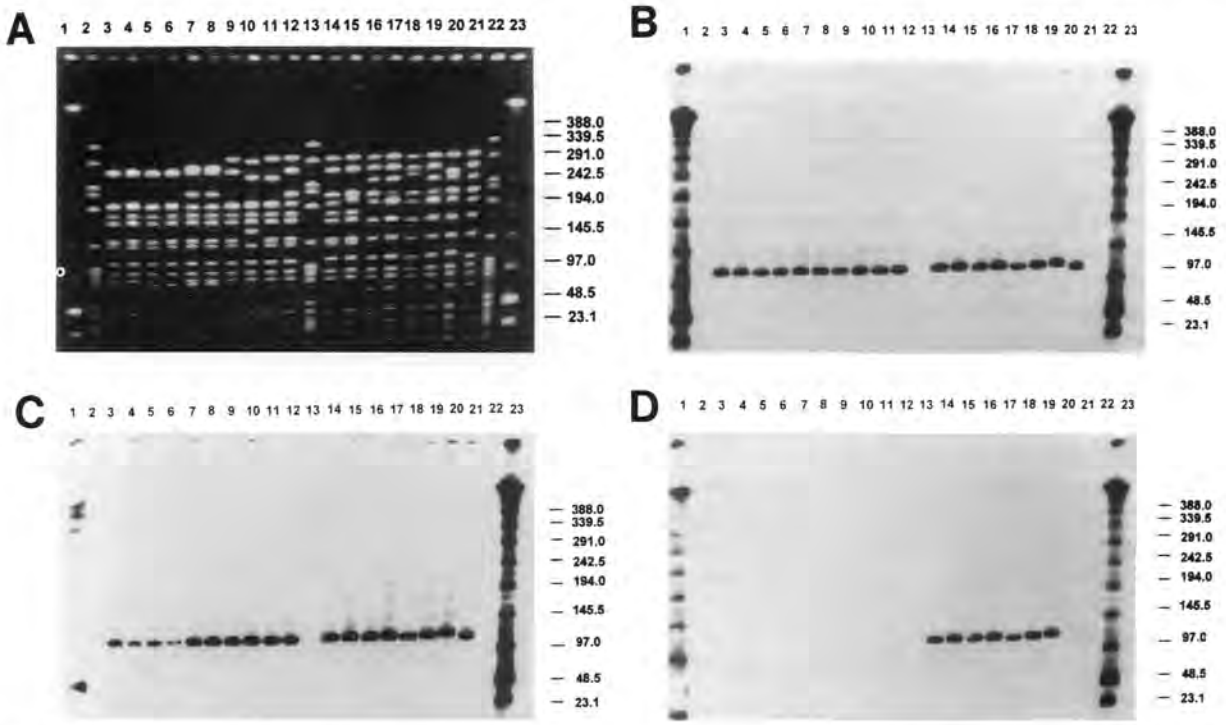

FIG. 3. PFGE and DNA hybridization patterns of penicillin-resistant *S. pneumoniae* isolates from the United States belonging to cluster 3. **(A)** PFGE patterns; **(B)** hybridization with *mefE*; **(C)** hybridization with *tetM*; **(D)** hybridization with *ermB*. Lanes 1 and 23, LMW ladder; lanes 2, 13, and 22, laboratory strain R6; lanes 3–12 and 14–21, strains CA14, TX4, CA37, CA40, CA41, CA38, DE2, NE11, TX25, TX8, CA44, TX13, CA22, TX20, AZ1, IN1, NE21, and TN8.

14, 14, and 6, respectively. Nevertheless, anomalous serotypes were also identified in most of the clusters.

DISCUSSION

High frequency of two penicillin-resistant internationally spread epidemic clones in the United States

The most interesting and revealing observation documented by the molecular fingerprinting techniques is the high proportion of two internationally spread clones of *S. pneumoniae* among penicillin-resistant strains recovered in the United States. The multiresistant Spanish/USA clone, usually expressing serotype 23F and referred to in this communication as clone A, represented close to 40% of all highly penicillin-resistant isolates from the United States. In the geographically diverse sampling used in this surveillance, clone A was detected among penicillin-resistant isolates from at least 30 of the 39 states providing samples (see Table 1 and Fig. 6A). In addition, this clone also made up the major proportion of all penicillin-resistant isolates examined from several states. For instance, clone A represented nearly half of the 54 penicillin-resistant isolates from California, 1/3 of the 37 isolates from Georgia, and about 2/3 of the penicillin-resistant isolates analyzed from the collection recovered in Florida and New York. The similar high representation of clone A among penicillin-resistant isolates from California and penicillin resistant isolates in Mexico is striking.[12] Wide geographic spread and high frequency was also a characteristic of a second international clone, referred to in this paper as clone B, which was identified in 18 of the states. The two clonal types together accounted for slightly over half of all the penicillin resistant bacteria from the United States.

Penicillin-resistant clusters

Besides the two international clones, a set of eight additional genetic lineages or clusters were also significant contributors to the collection of penicillin-resistant strains (see Table 2). A cluster was arbitrarily defined as a unique PFGE type shared by at least five independent isolates, and on the basis of these criteria we identified eight clusters, the largest one of which (cluster 18) was represented by 31 isolates. Out of the 328 penicillin-resistant strains examined, 112 (34.1%) were present as members of one of these eight clusters. Representatives of at least two of these clusters have already been identified in a 1994/1995 survey of penicillin-resistant *S. pneumoniae* from the United States,[9] but none of these particular PFGE types has been seen so far in molecular epidemiological studies performed outside the United States.

Similarly to clones A and B, each of the eight U.S. clusters was spread across state boundaries (see Fig. 6B). Most of the clusters also carried resistance traits against antibiotics other than penicillin and two of the clusters, namely clusters 5 and 18, included bacteria with extremely high penicillin MIC values (MIC = 16 μg/ml). In fact, in the case of cluster 18, the largest proportion of isolates had penicillin MIC values of 4

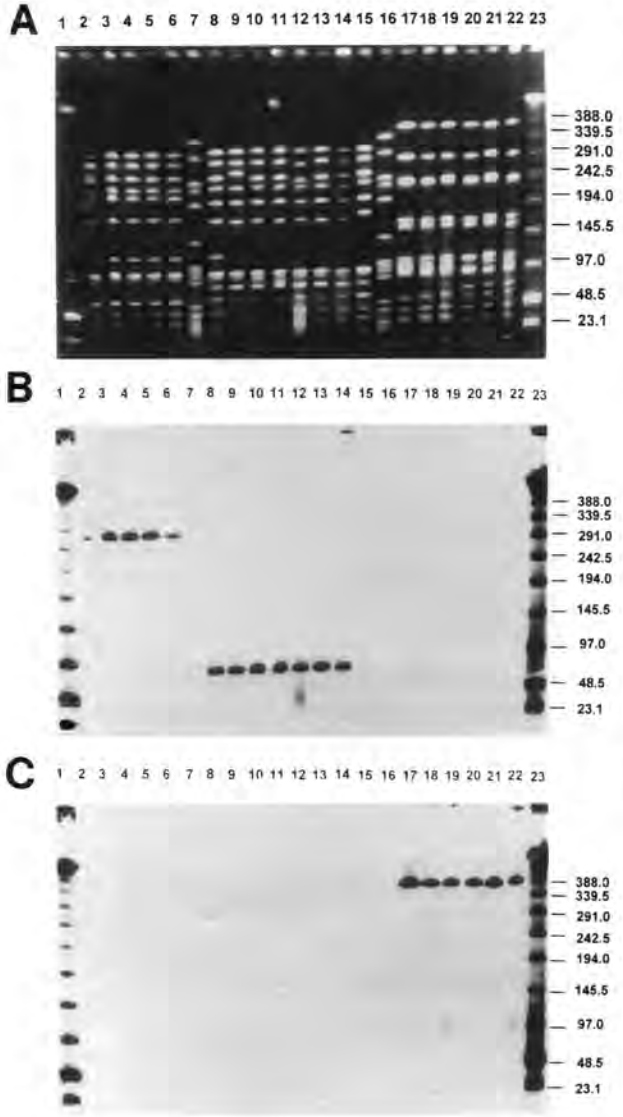

FIG. 4. PFGE and DNA hybridization patterns of *S. pneumoniae* isolates from the United States, belonging to clusters 8, 22, and 16. **(A)** PFGE pattern; **(B)** hybridization with *mefE*; **(C)** hybridization with *tetM*. Lanes 1 and 23, Lambda ladder; lanes 7–16, laboratory strain R6; lanes 2–6, cluster 8 strains ARK2, ARK1, MI1, SD1, and SD2; lanes 8–15, cluster 22 strains OH3, KY9, TX39, KY16, WI2, SD3, MD1, and DE1. MD1 (lane 14) is penicillin susceptible. Lanes 17–22 contain the cluster 16 strains TX5, GA30, TN12, KS10, TN13, and TN17.

μg/ml and a significant proportion of cluster 18 isolates had penicillin MICs between 6 and 12 μg/ml (see Fig. 7B).

Multiresistant strains with extremely high penicillin MIC values

Most of the isolates (179/328) had penicillin MICs of 2 μg/ml, with the next largest group (113 isolates) showing penicillin MICs of 4 μg/ml (see Fig. 7A and Table 4). Although the majority of the isolates belonging to clones A and B had penicillin MIC values of 2 μg/ml (see Fig. 7B), over 1/3 of clone A and clone B isolates had penicillin MICs of 4 μg/ml, and at least seven isolates belonging to clone A had penicillin MICs as high as 6 or 8 μg/ml. Also included in Fig. 7B is the distribution of penicillin MICs among the cluster 18 isolates. The most frequent penicillin MIC value in this cluster was 4 μg/ml, but other isolates showing MIC values as high as 12 and 16 μg/ml were also detected in this large pneumococcal lineage. This shift in the direction of increased penicillin MICs among *S. pneumoniae* isolates is of concern, particularly when such high MIC values are associated with epidemic strains such as the serotype 23F Spanish/USA clone or the serotype 9/14 French clone. Although *S. pneumoniae* strains with MIC values up to 2 μg/ml do not present complications to β-lactam chemotherapy in pneumonia,[24] this may no longer be the case with the highly resistant strains.

Resistance to antibiotics other than penicillin

Increase in antibiotic resistance among *S. pneumoniae* isolates from the United States is not restricted to penicillin. Surveys done in the mid- to late 1980s recorded erythromycin resistance in approximately 0.3% of pneumococcal isolates, and resistance to SXT was also limited to about 0.6% of the isolates.[31] Both of these figures increased substantially in recent surveys, which show erythromycin resistance in over 10% and SXT resistance in close to 20% of all isolates.[31] Data reported in this communication suggest that a considerable portion of resistance to antibiotics other than penicillin is actually linked to the spread of multidrug-resistant lineages of penicillin-resistant pneumococci. For instance, increase in the frequency of resistance to chloramphenicol, tetracycline, and macrolides appears to be linked to the frequent presence of one or more of these resistance traits in international clone A, and in the penicillin-resistant clusters identified in the U.S. sample.

Antibiotic resistance genes and resistance phenotypes

Among the 328 penicillin-resistant pneumococci from the United States, 212 strains (64.6%) were erythromycin resistant (see Table 4). Most of these bacteria (138/212) carried the *mefE* gene and expressed low-level macrolide resistance, and 65/212 carried the *ermB* gene and expressed high-level MLS-type resistance. Discrepancies between the macrolide resistance genes and resistant phenotype were also noted. For instance, in seven isolates expressing high-level erythromycin resistance, the bacteria carried both the *mefE* and *ermB* genes. A single isolate (WA3) showed erythromycin resistance of 8 μg/ml but hybridized with *ermB* instead of *mefE*. Another strain with low-level erythromycin resistance did not react with either one of the two macrolide resistance gene probes. Five isolates belonging to three different PFGE types were susceptible to erythromycin and yet gave positive hybridization signal with the *mefE* probe. Similar, anomalous macrolide resistance patterns have recently been detected among *S. pneumoniae* isolates from Canada.[15]

Out of the 328 penicillin-resistant U.S. strains examined, 141 chloramphenicol-resistant strains hybridized with the *cat* DNA probe, and 180 strains were resistant to tetracycline and reacted with the *tetM* DNA probe. An additional 36 isolates (most of them [29/36] belonging to cluster 18) were phenotypically sus-

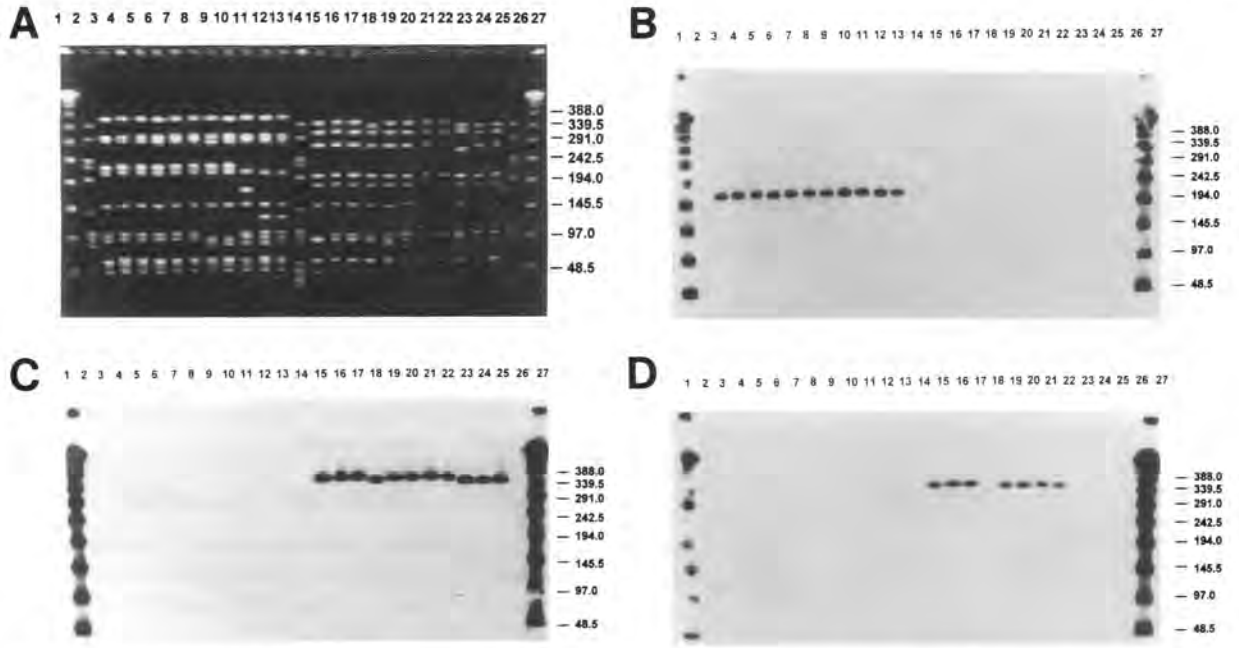

FIG. 5. PFGE and DNA hybridization patterns of penicillin-resistant *S. pneumoniae* isolates from the United States belonging to clusters 9 and 6. **(A)** PFGE patterns; **(B)** hybridization with *mefE*; **(C)** hybridizations with *tetM*; **(D)** hybridization with *cat*. Lanes 1 and 27, Lambda ladder; lanes 2, 14, and 26, laboratory strain R6; lanes 3–13, cluster 9 isolates OH4, OH11, OH12, OH13, OH14, OH19, CA30, FL28, GA8, GA21, and OH2; lanes 15–25, cluster 6 isolates GA10, GA28, GA36, CA9, KS3, GA5, GA16, GA33, GA19, GA27, and GA26.

ceptible to tetracycline but still gave strong hybridization with the *tetM* DNA probe (see Fig. 2C).

Contrasts and similarities between the molecular profiles of penicillin-resistant S. pneumoniae *from Latin America and the United States*

A recent evaluation of penicillin-resistant *S. pneumoniae* isolates from six Latin American countries[35] allows some interesting comparisons between the Latin American and U.S. samples. Among the 328 resistant strains characterized from the United States, the multiresistant clone A (most frequently carrying resistance to SXT, chloramphenicol, tetracycline, and erythromycin) was present in 38.7% of the isolates, while international clone B, resistant to penicillin and SXT only, was represented by 12.2% of the isolates. The frequency of these two clones was inverse among the 172 penicillin-resistant Latin American *S. pneumoniae* collection in which clone A made up 25.6%, and clone B 55.2% of the isolates.[35] This seems to explain, at least in part, the substantially lower frequency of chloramphenicol, tetracycline, and macrolide resistance in the Latin American sample. In addition, clone A, recovered from the United States, frequently carried the erythromycin resistance trait (97/127 isolates), while macrolide resistance was relatively rare (4/44, or 9.1%) among clone A isolates from Latin America. Interestingly, the four erythromycin-resistant strains from the Latin American sample were recovered in Mexico,[12] *i.e.*, close to the U.S. border.

An additional difference was noted when comparing serotypes expressed by members of international clone B recovered in the United States as compared to the same clone type recovered from the Latin American surveillance: The overwhelming majority of the clone B U.S. strains expressed serotype 9 (35/40), whereas most of the Latin American clone B isolates expressed serotype 14 (87/95).[35]

Evidence for frequent capsular switch

The majority of isolates belonging to the two international clones and to the eight distinct resistance clusters each expressed a unique capsular serotype (see Table 2). However, atypical serotypes were also detected with surprisingly high frequency, particularly among members of international clone A where close to 30% of all isolates (39/127) expressed capsular serotypes other than the typical 23F, namely, serotypes 19 in 37 of the isolates, one serotype 14 isolate, and one nontypable. Serotype switch was also observed among the 40 isolates representing clone B in which five of the predominantly serotype 9 isolates expressed either serotype 14 (two isolates), serotype 19 (one isolate) or were nontypable (two isolates). A spontaneous switch in capsular serotype has been described repeatedly in the recent literature,[1,6,23] and in a disproportionately large number of reported cases the apparent recipients of new capsular type were members of the 23F Spanish/USA international clone.[26] While in most of these cases the newly acquired serotypes were other "pediatric" serotypes (19,14,9), in at least one case the switch involved acquisition of serotype 3, which was also accompanied by a large increase in virulence.[23] Genetic studies indicate that such capsular switch events include exchange of large pieces of DNA, possibly through genetic transformation *in vivo*.[25,27]

TABLE 2. RESISTANCE PHENOTYPES AND GENOTYPES OF *S. PNEUMONIAE* BELONGING TO CLONES A, B, AND CLUSTERS

	Clone A	*Clone B*	*Cluster 18*	*Cluster 3*	*Cluster 5*	*Cluster 6*	*Cluster 9*	*Cluster 22*	*Cluster 16*	*Cluster 10*
Number of isolates	**127**	**40**	**31**	**20**	**16**	**13**	**13**	**7**	**7**	**5**
Prototype resistance pattern	**PETCX**	**PX**	**PEX**	**PETX**	**PX**	**PETCX**	**PEX**	**PEX**	**PETX**	**PETCX**
CMP R (*cat*+)	125	1	0	0	0	9	0	0	0	4
CMP S (*cat*−)	2	39	31	20	16	4	13	7	7	1
TET R (*tetM*+)	123	2	0	20	0	11	1	0	7	5
TET S (*tetM*−)	0	38	2	0	16	2	12	7	0	0
TET S (*tetM*+)	4	0	29	0	0	0	0	0	0	0
ERY R Total	97	7	30	20	0	11	11	6	7	5
ERY R Low (*mefE*+)	59	6	30	13	0	0	10	6	0	0
ERY R High (*ermB*+)	37	0	0	0	0	11	1	0	7	5
ERY R High (*mefE*+ and *ermB*+)	0	0	0	7	0	0	0	0	0	0
ERY R Low (*ermB*+)	1	0	0	0	0	0	0	0	0	0
ERY R Low (*mefE*− and *ermB*−)	0	1	0	0	0	0	0	0	0	0
ERY S (*mefE*− and *ermB*−)	28	33	1	0	15	2	0	1	0	0
ERY S (*mefE*+)	2	0	0	0	1	0	2	0	0	0
SXT R	127	40	31	20	16	13	13	6	7	5
LEV R	4	1	0	0	0	0	0	0	0	0
PEN MIC_{50} (μg/ml)	2	2	4	2	3	2	2	4	4	2
PEN MIC_{90} (μg/ml)	4	3	12	4	8	3	2	8	6	4
PEN MIC range (μg/ml)	1.5–8.0	1.5–4.0	1.5–16	1.5–6.0	1.5–16	1.5–4	1.5–3	2–8	3–8	1.5–6

R, resistant; S, susceptible; P/PEN, penicillin; E/ERY, erythromycin; T/TET, tetracycline; C/CMP, chloramphenicol; X/SXT, trimethoprim/sulfamethoxazole; LEV, levofloxacin; Low erythromycin MIC 1–32 μg/ml; High erythromycin MIC $\geq$ 256 μg/ml.

TABLE 3. DISTRIBUTION OF SEROTYPES AND PFGE TYPES AMONG PENICILLIN-RESISTANT *S. pneumoniae* ISOLATES FROM THE UNITED STATES

	Number of isolates												
	International clones		*Clusters*								*Number of isolates*		*Total number of isolates (%)*
Serotype	*A*	*B*	*18*	*3*	*5*	*6*	*9*	*22*	*16*	*10*	*Int. clones + clusters*	*Other PFGE types*	
23	88	—	—	—	15	—	—	—	—	—	103	8	111 (33.8)
6	—	—	30	—	1	12	12	—	—	5	60	17	77 (23.5)
19	37	1	—	19	—	—	—	—	—	—	57	2	59 (18.0)
9	—	35	—	—	—	—	—	—	—	—	35	2	37 (11.3)
14	1	2	—	1	—	—	—	7	6	—	17	—	17 (5.2)
NT	1	2	1	—	—	1	1	—	1	—	7	20	27 (8.2)
Total	127	40	31	20	16	13	13	7	7	5	279	49	328
(%)	(38.7)	(12.2)	(9.4)	(6.1)	(4.9)	(4.0)	(4.0)	(2.1)	(2.1)	(1.5)	(85.0)	(15.0)	(100)

Appearance of levofloxacin-resistant S. pneumoniae

None of the 366 penicillin-resistant *S. pneumoniae* isolates from the recent Latin American surveillance study showed resistance to ofloxacin.[35] Similarly, resistance to levofloxacin was extremely rare in the U.S. sample: a total of only 20 resistant strains could be identified in the entire collection of 9,160 *S. pneumoniae* isolates (both penicillin-susceptible and penicillin-insusceptible) collected in the TRUST Study.[34] A high proportion of the resistant strains (16/20, or 80%) expressed high-level levofloxacin resistance (MIC = 32 μg/ml); MICs in the rest of the isolates were between 3 and 4 μg/ml. Most of the levofloxacin-resistant strains were genetically heterogeneous, as shown by the diverse PFGE types (see Fig. 1D). Seven of the levofloxacin-resistant isolates were in penicillin-susceptible pneumococci, six in intermediately penicillin-resistant isolates, and seven in highly penicillin-resistant strains, the latter of which included four isolates that belonged to international clone A and one isolate that shared properties of international clone B. The appearance of fluoroquinolone resistance among these two highly epidemic strains is of obvious concern and requires careful surveillance.

The extensive genetic heterogeneity shown by most of the 20 levofloxacin-resistant *S. pneumoniae* isolates indicates that resistance to this relatively recently introduced antibiotic is emerging through a *de novo* mechanism, presumably through the introduction of point mutations into the gyrase genes selected for by antibiotic pressure. Whether resistance to levofloxacin will continue to spread through such a *de novo* mechanism or through the spread of the relatively few strains belonging to multiresistant epidemic lineages that have already acquired levofloxacin resistance cannot be predicted at the present time.

Mechanism of spread of penicillin-resistant S. pneumoniae *in the United States*

The epidemiological factor(s) responsible for the abrupt increase in frequency of penicillin-resistant strains in the United States from a figure of about 4–5% in the 1980s, to 25% in the early 1990s, and to 35–40% recorded in the most recent surveys, is not understood. Parallel with the increase in frequency of penicillin-insensitive pneumococci there was also a disproportionately large increase in the proportion of penicillin-resistant strains, which increased from a 0.1–0.2% figure in the 1980s, to between 7 and 9% in the early 1990s, and to about 15% of all the penicillin-insensitive isolates in the most recent surveys. The observations described in this communication

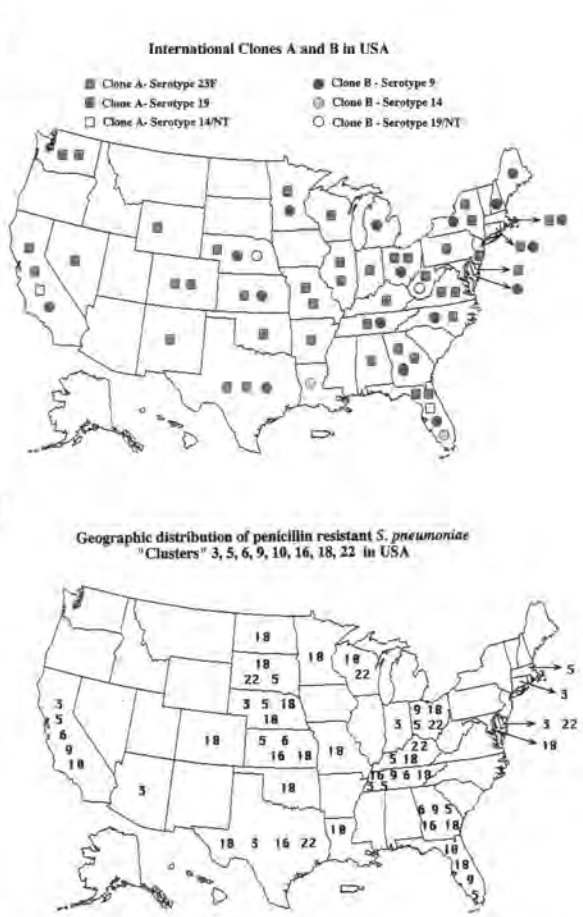

FIG. 6. (**A**) Geographic distribution of international clones A and B and (**B**) the penicillin-resistant *S. pneumoniae* clusters in the United States.

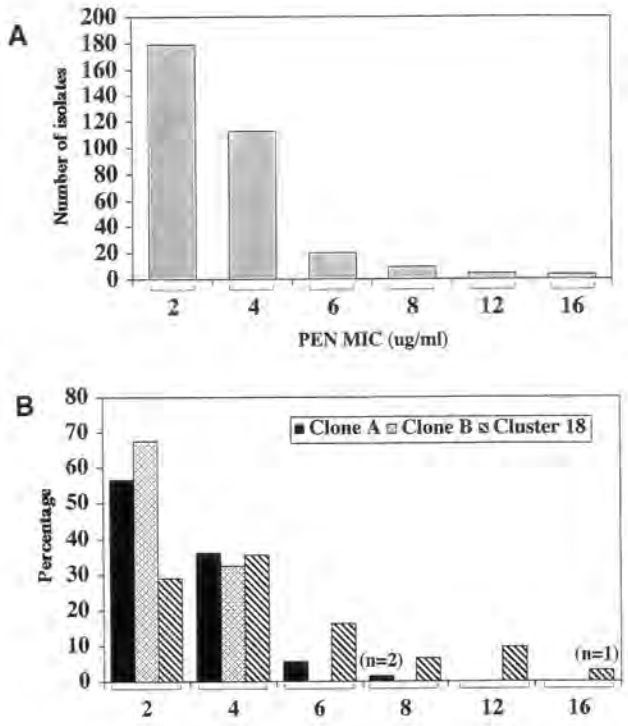

FIG. 7. Distribution of penicillin MICs among 328 penicillin-resistant *S. pneumoniae* isolates from the United States (**A**) and frequency of Clones A and B and Cluster 18 isolates with different penicillin MICs (**B**).

strongly suggest that a major mechanism involved with this process was the importation of two pandemic penicillin-resistant and multiresistant clones from abroad and the extensive geographic spread of these bacteria within the United States.

Additional significant contributors to the increased frequency of penicillin resistance are the eight uniquely U.S. genetic lineages, or clusters, identified by PFGE type, serotype, antibiotic resistance profiles and resistance genes described in this communication. These eight clusters, together with the two international clones, accounted for 85% of all the penicillin-resistant pneumococci. In the sample we analyzed, only a relatively small portion (49/328 or 15%) of penicillin-resistant strains were represented by mainly solitary isolates with diverse genetic backgrounds (28 distinct PFGE types in 49 strains).

The extensive spread of the resistant clones and clusters across state boundaries and across virtually the entire country must, at least in part, be a reflection of the social mobility of the human population of the United States. The Spanish/USA clone and the French clone are also major contributors to penicillin-resistant *S. pneumoniae* in six Latin American countries,[3–5,28,35] Italy,[19] Bulgaria,[29] France,[13,18] Portugal,[37] and East Asia.[21,33] Microbiological factors, if any, associated with the epidemicity of these clones, remain to be identified.

The relationship between the predominantly low-level penicillin-resistant pneumococci from the 1980s and the highly penicillin-resistant strains that have become more abundant in the recent U.S. surveys is not clear. The low-level resistant strains preceding chronologically the highly resistant pneumo-

TABLE 4. RESISTANCE PHENOTYPES AND GENOTYPES OF *S. PNEUMONIAE* ISOLATES FROM THE UNITED STATES

	Clones A and B and clusters *No. (%)*	*Other PFGE types* *No. (%)*	*All PFGE types* *No. (%)*
TOTAL No. (% of total)	**279 (85)**	**49 (15)**	**328 (100)**
Prototype resistant pattern	**PET(C)X**	**PX**	**P(ETC)X**
CMP R (*cat+*)	139 (**49.8**)	2 (**4.1**)	141 (**43**)
CMP S (*cat−*)	140 (50.2)	47 (95.9)	187 (57)
TET R (*tetM+*)	169 (**60.6**)	11 (**22.4**)	180 (**54.9**)
TET S (*tetM−*)	77 (27.6)	35 (71.4)	112 (34.1)
TET S (*tetM+*)	33 (11.8)	3 (6.1)	36 (11)
ERY R Total	194 (**69.5**)	18 (**36.7**)	212 (**64.6**)
ERY R Low (*mefE+*)	124 (44.4)	14 (28.6)	138 (42.1)
ERY R High (*ermB+*)	61 (21.8)	4 (8.2)	65 (19.8)
ERY R High (*mefE+* and *ermB+*)	7 (2.5)	0 (0)	7 (2.1)
ERY R Low (*ermB+*)	1 (0.3)	0 (0)	1 (0.3)
ERY R Low (*mefE−* and *ermB−*)	1 (0.3)	0 (0)	1 (0.3)
ERY S (*mefE−* and *ermB−*)	80 (28.7)	31 (63.3)	111 (42.1)
SXT R	278 (**99.6**)	39 (**79.6**)	317 (**96.6**)
LEV R	5 (**1.8**)	2 (**4.1**)	7 (**2.1**)
PEN MIC_{50} (μg/ml)	2	2	2
PEN MIC_{90} (μg/ml)	6	4	6
PEN MIC range (μg/ml)	1.5–16	1.5–8	1.5–16

No., Number of isolates; R, resistant; S, susceptible; P/PEN, penicillin; E/ERY, erythromycin; T/TET, tetracycline; C/CMP, chloramphenicol; X/SXT, trimethoprim/sulfamethoxazole; LEV, levofloxacin; Low, erythromycin MIC 1–32 μg/ml; High, erythromycin MIC $\geq$ 256 μg/ml.

cocci may have emerged locally "*de novo*" from penicillin-susceptible ancestral pneumococci that have acquired penicillin resistance traits. Current models assume acquisition of such resistance traits through heterologous recombinational events leading to the formation of "mosaic" pbp genes.[11] Such *de novo* emergence of penicillin resistance may have been at the origin of some of the U.S. clusters. For instance, in addition to the 16 cluster 5 isolates, a penicillin-susceptible strain, WA2 (penicillin MIC of 0.02 μg/ml) also shared the distinct PFGE type of this cluster, while other members of this lineage included bacteria with penicillin MIC values of 2, 6, and as high as 16 μg/ml. A similar case was observed among the serotype 14 isolates belonging to cluster 22: In addition to the seven isolates with high penicillin MICs of 4–8 μg/ml, cluster 22 also included a single strain, MD1, which was susceptible to penicillin (MIC of 0.04 μg/ml). The two penicillin-susceptible strains of these two clusters may represent the genetic backgrounds of the ancestral cells from which these two particular resistant lineages emerged. The fact that the two clusters include both susceptible as well as extremely highly penicillin-resistant isolates will make these strains useful in studies on the mechanism(s) by which penicillin MICs are increased in *S. pneumoniae*.

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