JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2002, p. 3660–3665 Vol. 40, No. 10
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.10.3660–3665.2002

Genotypes of Invasive Pneumococcal Isolates Recently Recovered from Italian Patients

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Received 28 May 2002/Returned for modification 21 June 2002/Accepted 12 July 2002

We examined 73 recent invasive pneumococcal isolates within selected areas of Italy for genotypic variability. Thirty-three genomic macrorestriction types were found, three of which represented multiple serotypes. Restriction fragment patterns of *pbp2b*, *pbp2x*, and *pspA* were conserved within the majority of isolates that shared macrorestriction types. Of the nine macrorestriction types found among the 22 penicillin-nonsusceptible *Streptococcus pneumoniae* (PNSP) isolates, seven comprised isolates with allelic profiles showing five to seven allelic matches to profiles in the multilocus sequence typing database (www.mlst.net); however, three of the seven profiles represented serotypes not previously associated with these clonal clusters. Two PNSP macrorestriction types represented new clones with unique allelic profiles. Allelic profiles obtained from isolates of 3 of the 25 macrorestriction types found among the 51 penicillin-susceptible *S. pneumoniae* (PSSP) isolates were closely related to previously described profiles. One PSSP isolate was a novel type 24F isolate related to the multiresistant clone France^{9V}-3. This work reports new PNSP strains and new serotype-clone associations.

Streptococcus pneumoniae is a major cause of community-acquired infections ranging from severe otitis media and sinusitis to pneumonia, bacteremia, and meningitis (2). Strains of serogroups 6, 9, 14, 19, and 23 have demonstrated a remarkable propensity for horizontal recombination events leading to beta-lactam resistance and capsular serotype switching (3, 4). Although capsular serotype switching has been widely reported among penicillin-resistant isolates, it has not been extensively investigated among penicillin-susceptible isolates.

Penicillin-binding protein (PBP) gene restriction profiles and sequences are often predictive of penicillin susceptibility or resistance, and together with *pspA* restriction profiles, they provide genetic information that supplements genomic profile analysis. Dissimilarity at these hypervariable loci among genomically related isolates potentially provides evidence of recent recombination events. In addition, PspA proteins are strong vaccine candidates (7, 10), and *pspA* sequence information from common strains in different countries could provide information relevant for future vaccine-related studies.

Infection due to non-penicillin-susceptible *S. pneumoniae* (PNSP) in Italy is still relatively uncommon, occurring at a frequency of about 10% (13). Genetic relatedness among pneumococcal isolates recovered in Italy has been surveyed only in one collection of noninvasive pneumococci with reduced susceptibility to penicillin (9). The present study describes genotypic analysis of 73 invasive isolates recovered in Italy, including both penicillin-sensitive and penicillin-nonsusceptible strains.

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(This work was presented in part as a poster at the 12th European Congress of Clinical Microbiology and Infectious Diseases, Milan, Italy, 21 to 24 April 2002 [abstr. P446].)

MATERIALS AND METHODS

Pneumococcal isolates. Sterile-site isolates from patients with bacteremia (28 isolates) or meningitis (45 isolates) were recovered primarily during the years 1998 to 2000 (59 isolates). Invasive isolates were collected from cases of meningitis during a large nationwide study in Italy (13). Bacteremic pneumococcal strains were obtained from patients in three cities: Bergamo (north Italy), Rome (central Italy), and Naples (south Italy). Twenty-two isolates for which penicillin MICs were ≥0.125 μg/ml were classified as PNSP. Eighteen of the 22 invasive PNSP isolates were recovered from patients with meningitis, and 4 were from patients with bacteremia. Fifty-one invasive penicillin-susceptible *S. pneumoniae* (PSSP) isolates (penicillin MICs, <0.125 μg/ml) were randomly chosen mainly from the centers in Bergamo and Naples, with 27 recovered from meningitis patients and 24 isolated from bacteremia patients.

PFGE profiles from strains Spain^{6B}-2, France^{9V}-3, Tennessee^{23F}-4, England^{14}-9, Spain^{14}-5, Hungary^{19A}-6, South Africa^{19A}-7, South Africa^{6B}-8, Slovakia^{14}-10, and Slovakia^{19A}-11 (11) were used for comparison.

Identification and serotyping. Pneumococcal strains were identified by optochin susceptibility and bile solubility. All isolates were serotyped using the Pneumotest panel (Statens Seruminstitut, Copenhagen, Denmark). Serotypes not included in the panel were identified at the Statens Seruminstitut.

Antibiotic susceptibility testing. Antibiotic susceptibility tests were performed using the E-test (AB Biodisk, Solna, Sweden). Susceptibilities to penicillin, ceftriaxone, erythromycin, chloramphenicol, clindamycin, and tetracycline were determined according to published guidelines (12).

PFGE. *Sma*I macrorestriction patterns were assigned designations as previously described (6). Isolates differing by 1 to 6 bands from subtype 1 of each type were assigned to the same type. Isolates with identical pulsed-field gel electrophoresis (PFGE) profiles were assigned to the same subtype. Isolates differing by more than 6 bands were classified as different PFGE types.

MLST. Thirteen isolates were subjected to multilocus sequence typing (MLST) as previously described (8).

PCR, restriction profiling, and *pspA* sequencing. PBP gene amplicon restriction digest profiling, *pspA* amplicon restriction digest profiling, and *pspA* sequencing were performed as previously described (1, 6).

TABLE 1. Serotypes, place of isolation, antibiotic resistance patterns, PFGE types, and *pbp2b*/*pbp2x*/*pspA* restriction patterns of pneumococcal isolates

PFGE subtype(s)	Serotype (no. of isolates)[a]	Place of isolate recovery (yr[s][b])	MIC range (μg/ml)		*pbp2b*/*pbp2x*/*pspA* restriction patterns	Gen Bank accession no. of closest *pspA* CDR[c] sequence (% identity; clade)	Antibiotype(s)[d] (in order of frequency)
			Penicillin	Ceftriaxone			
1.1, 1.3, 1.5, 1.6	9V (5), France^{9V}-3	Arezzo, Naples (97, 99, 00)	0.5–2	0.5–1	6/2/1, 6/2/2, 6/2/3	AF252286 (100:3)	PG; PG, TX, EM, CC, TC
1.2	9A (1)	Naples (00)	1	0.5	6/2/1	ND[e]	PG, EM
1.4	14 (1)	Bergamo (99)	2	0.5	6/2/1	ND	PG
1.7	24F (1)	Emilia-Romagna (98)	0.03	0.03	2/8/1	AF252286 (100; 3)	No resistance
2.1	19A (3)	Naples (98, 99)	0.12	0.03–0.12	10/27/4	AF253408 (100; 4)	PG, EM, CC, TC; PG, EM, CC
2.1	15A (1)	Palermo (98)	0.25	0.12	10/27/4	AF253408 (100; 4)	PG, EM, CC, TC
3.1	24F (3)	Naples (97, 98, 00)	0.5	0.06–0.25	27/8/8	AF253407 (100; 1)	PG, EM, CC, TC
4.1, 4.2	6B (2)	Parma, Bergamo (98, 99)	0.12	0.06	12/22/6 12/22/7	AF071805 (100; 1) AF254255 (99; 5)	PG, EM, CC, TC
5.1, 5.2	35F (2)	Naples, Rome (99, 00)	0.12–0.25	0.06–0.25	5/4/11	AF071806 (98; 1)	PG
6.1	6A (1)	Rome (98)	0.5	≤0.012	28/12/negative	ND	PG, EM, CC, TC
7.1	19F (1)	Bologna (99)	0.5	0.25	29/28/5	AF252286 (100; 3)	PG, EM, CC
8.1	23F (1)	Naples (99)	2	1	30/9/9	AF288751 (100; 5)	PG, TX, EM, CC, TC
9.1	19F (1)	Naples (00)	0.5	0.12	negative/29/10	AF071812 (100; 2)	PG, CH, EM, CC, TC
10.1–10.5	23F (7)	Bergamo, Como, Monza, Pordenone (97, 98, 99, 00)	≤0.03	≤0.12	2/8/12, 2/30/12, 2/8/17 AF253407 (100; 1)	AF253407 (100; 1)	No resistance
11.1–11.6	14 (7), England^{14}-9	Bergamo, Monza, Rome (98, 99)	≤0.03	≤0.25	1/1/19	AF253406 (100; 1)	EM, CC, TC; EM, CC; EM; TC
12.1, 12.2	3 (4)	Bergamo, Milan, (87, 90, 98, 99)	≤0.03	≤0.03	1/1/9, 2/8/9	AF288751 (100; 5)	No resistance
13.1–13.3	12F (5)	Naples, Milan, Florence (98)	≤0.03	≤0.03	1/1/13	AF255552 (97; 2)	No resistance
14.1	8 (2)	Ferrara, Milan (98)	≤0.03	≤0.03	2/1/29	AF253408 (93.5; 4)	No resistance
15.1–15.3	4 (3)	Bergamo, Naples, Feltre (91, 98, 00)	≤0.03	≤0.03	2/8/31	ND	No resistance
15.4	18C (1)	Bergamo (00)	≤0.03	≤0.03	2/1/31	ND	No resistance
16.1, 16.2	7F (2)	Bergamo (99)	≤0.03	≤0.03	2/1/32	AF255545 (100; 2)	No resistance
17.1, 17.2	9V (2)	Bergamo (90, 99)	≤0.03	≤0.03	1/1/20	ND	No resistance
18.1	23A (1)	Naples (98)	≤0.03	≤0.03	2/8/25	ND	No resistance
18.2	23F (1)	Naples (98)	≤0.03	≤0.03	1/8/25	ND	EM, CC, TC
19.1	23F (1)	Monza (98)	≤0.012	≤0.012	1/1/14	ND	No resistance
20.1	23F (1)	Bergamo (99)	≤0.03	≤0.012	1/8/15	ND	No resistance
21.1	19A (1)	Bergamo (99)	≤0.03	≤0.012	1/1/24	ND	No resistance
22.1	10A (1)	Bergamo (96)	≤0.012	0.03	2/1/28	ND	No resistance
23.1	9V (1)	Bergamo (99)	≤0.03	0.03	2/1/23	ND	TC
24.1	5 (1)	Bergamo (96)	≤0.03	≤0.012	1/1/21	ND	No resistance
25.1	19F (1)	Mantova (98)	≤0.012	≤0.012	2/1/26	ND	No resistance
26.1	1 (1)	Merate (98)	≤0.012	≤0.012	2/1/27	ND	No resistance
27.1	1 (1)	Rome (97)	≤0.012	≤0.012	2/8/16	ND	No resistance
28.1	33 (1)	Bergamo (91)	≤0.03	≤0.03	2/1/30	ND	No resistance
29.1	22F (1)	Naples (98)	≤0.012	≤0.03	2/1/22	ND	No resistance
30.1	1 (1)	Bergamo (91)	≤0.012	≤0.03	2/1/33	ND	No resistance
31.1	10A (1)	Bergamo (90)	0.03	≤0.012	2/1/12	ND	No resistance
32.1	4 (1)	Naples (98)	≤0.012	0.03	2/8/34	ND	No resistance
33.1	6B (1)	Bergamo (99)	0.03	0.03	2/26/18	ND	TC

[a] Reference strains France^{9V}-3 and England^{14}-9 shared identical genetic profiles with PFGE subtype 1.1 and PFGE subtype 11.1 isolates, respectively.
[b] Year designations are to be understood as preceded by "19" except for the designation 00, representing 2000.
[c] CDR, clade-defining region.
[d] PG, penicillin; TX, ceftriaxone; EM, erythromycin; CC, clindamycin; TC, tetracycline; CH, chloramphenicol.
[e] ND, not determined.

Nucleotide sequence accession numbers. GenBank accession numbers for the partial *pspA* sequences are AF490265 for the PFGE type 4.2, serotype 6B isolate, AF490266 for the PFGE type 5, serotype 35F isolates, AF490267 for the PFGE type 13, serotype 12F isolates, and AF490268 for the PFGE type 14, serotype 8 isolates.

RESULTS

Antibiotic resistance. Eighteen isolates were intermediately resistant to penicillin, and four isolates were resistant to higher levels of penicillin. These 22 isolates included 6 isolates of serotypes that rarely include non-β-lactam-susceptible isolates (15A, 24F, and 35F) (Table 1). One type 23F isolate and one type 9V isolate were resistant to low levels of ceftriaxone. Fourteen PNSP (64%) and five PSSP (10%) isolates were found to be resistant to at least two antibiotics other than β-lactams. Twenty-five isolates were resistant to various combinations of the antibiotics erythromycin, clindamycin, and tetracycline. One multidrug-resistant isolate was resistant to chloramphenicol.

One set of seven type 23F isolates (PFGE type 10) had the unusual phenotype of penicillin sensitivity and reduced susceptibility to ceftriaxone. Additionally, one isolate (PFGE subtype 6.1) had the novel phenotype of intermediate penicillin resistance and a wild-type basal ceftriaxone MIC. For all isolates that we had observed previously for which penicillin MICs were ≥0.5 μg/ml, MICs of cephalosporins were higher than the basal levels seen for sensitive pneumococcal isolates (data not shown).

TABLE 2. Allelic profiles (MLST), capsular serotypes, PFGE types, and penicillin MICs for selected invasive isolates from Italy, 1997 to 2000, compared with most related sequence types previously reported at www.mlst.net

Isolate[a]	PFGE subtype	Serotype	MLST type	Penicillin MIC (μg/ml)	Allele at:						
					aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*dd1*
PN131	1.7	24F	162	0.032	7	11	10	1	6	8	14
United Kingdom	ND[b]	9V	162	0.012	7	11	10	1	6	8	14
United Kingdom	ND	14	162	0.5	7	11	10	1	6	8	14
United Kingdom	ND	19F	162	0.012	7	11	10	1	6	8	14
PN141	1.1	9V	ND	0.5	ND	ND	ND	ND	ND	ND	ND
France^{9V}-3	1.1	9V	156	2.0	7	11	10	1	6	8	1
PN81	2.1	15A	63	0.25	2	5	36	12	17	21	14
PN84	2.1	19A	63	0.12	2	5	36	12	17	21	14
Spain	ND	15A	63	0.12	2	5	36	12	17	21	14
PN76	3.1	24F	230	0.5	12	19	2	17	6	22	14
Denmark	ND	14	230	0.12	12	19	2	17	6	22	14
PN126	4.1	6B	315	0.12	20	28	1	1	15	14	14
SP95	4.2	6B	315	0.12	20	28	1	1	15	14	14
Poland	ND	6B	315	0.12	20	28	1	1	15	14	14
SP356	5.1	35F	Cdc40[c]	0.25	7	5	4	1	6	1	79
Spain	ND	6B	136	0.25	7	5	4	1	6	20	46
PN130	6.1	6A	Cdc41[c]	0.5	2	13	9	16	6	1	62
PN150	7.1	19F	Cdc42[c]	0.5	11	19	57	17	6	22	75
SP6	8.1	23F	242	2	15	29	4	21	30	1	14
Taiwan^{23P}-15	ND	23F	242	1.5	15	29	4	21	30	1	14
SP265	9.1	19F	88	0.5	5	5	7	7	8	5	7
Spain	ND	19F	88	0.12	5	5	7	7	8	5	7
102	10.1	23F	Cdc43[c]	≤0.012	1	8	6	2	34	20	6
Tennessee^{23F}-4	36	23F	37	0.12	1	8	6	2	6	4	6
PN99	11.2	14	15	0.032	1	5	4	5	5	3	8
United Kingdom	ND	14	15	0.012	1	5	4	5	5	3	8
England^{14}-9	11.1	14	9	0.012	1	5	4	5	5	1	8

[a] Entries from www.mlst.net are indicated by the country of origin listed at that site. All data for these strains are taken directly from this site.
[b] ND, not determined.
[c] Temporary allelic profile designations, since these profiles are not yet listed at http://www.mlst.net.

PFGE types found among PNSP isolates. Nine PFGE types were found among the 22 PNSP isolates. Among the 22 PNSP isolates, five PFGE types (types 1 to 5) accounted for 18 isolates (Table 1). Four of these five PFGE types represented multiple serotypes or serotypes rarely associated with a lack of susceptibility to penicillin. PFGE type 1 included the internationally disseminated multiresistant strain France^{9V}-3 (11) and isolates of four different serotypes, including one penicillin-sensitive type 24F isolate (isolate PN131 [Tables 1 and 2]). PFGE type 2 included four isolates representing two different serotypes, 19A and 15A. Three invasive serotype 24F isolates belonged to PFGE type 3, and PFGE type 5 consisted of two serotype 35F isolates. More-detailed information on the three invasive serotype 24F isolates has been published elsewhere (14).

PFGE types found among PSSP isolates. Among the 51 PSSP isolates, 25 PFGE types were found, encompassing 18 different serotypes. Fifteen unique PFGE types containing single strains (PFGE types 19 to 33) were observed among PSSP isolates. Nine PFGE types comprised multiple (2 to 8) isolates and accounted for 35 (69%) of the 51 PSSP isolates (Table 1). Only two of these types are discussed here. PFGE type 11 included seven serotype 14 isolates, as well as the reference clone England^{14}-9 (Table 1) (11). As with England^{14}-9, six of these isolates were erythromycin resistant, and all seven were sensitive to penicillin and ceftriaxone. Unlike England^{14}-9, a subset of these strains were resistant to clindamycin and/or tetracycline. PFGE type 15 consisted of four isolates representing two different serotypes, 4 and 18C.

MLST analysis. PFGE results were used to select 13 individual isolates for MLST analysis. These included representatives of each of the nine PFGE types found among the 22 PNSP isolates (Table 2). Isolates with serotype-PFGE type associations that we had not observed previously were preferentially chosen for analysis. Two isolates representing two PFGE types found only in PSSP isolates were also chosen for MLST analysis. Those types, PFGE types 10 and 11, each comprised seven or more independent isolates. Within PFGE type 1, shared with the international multidrug-resistant France^{9V}-3 clone, the allelic profile of the single serotype 24F antibiotic-sensitive isolate (PN131) was obtained. Its profile, ST162, proved to be a single-allele variant of the France^{9V}-3 allelic profile (ST156). According to the MLST database (available at www.mlst.net), ST162 has previously been found in both PSSP and PNSP isolates of different serotypes (9V,14, and 19F) isolated in the United Kingdom and Canada.

Two representative isolates (serotypes 15A and 19A) of PFGE type 2 were found to share allelic profile ST63, previously found in invasive serotype 15A isolates recovered in Spain.

One of the three PFGE type 3 intermediately penicillin resistant serotype 24F isolates was found to have allelic profile ST230, previously recorded for an intermediately penicillin

resistant invasive serotype 14 isolate recovered in Denmark in 1996 (14).

Two PFGE type 4 isolates, both intermediately penicillin resistant and belonging to serotype 6B, were found to share allelic profile ST315 with multiple invasive, intermediately penicillin resistant serotype 6B isolates recovered in Poland in 1996 (Table 2). It was interesting that the two profile ST315 isolates from Italy had quite divergent *pspA* sequences that were not of the same clade (interclade PspA proteins differ by >20% within the 100-residue clade-determining regions [7]).

PFGE type 5 was shared by two intermediately penicillin resistant serotype 35F isolates. MLST analysis of one of these isolates revealed that it was a new two-locus variant of ST136 (temporarily designated Cdc40). ST136 was previously found within an intermediately penicillin resistant serotype 6B meningitis isolate recovered in Spain. Interestingly, the *ddl* allele in allelic profile ST136 is a type B allele, which is found only among PNSP isolates (5), while the *ddl* allele from the type 35F, profile Cdc40 isolate was a newly discovered type A allele. Type B alleles display a wide degree of variation from *ddl* alleles found solely within sensitive strains; presumably, they originated among pneumococci through cotransformation with *pbp2b* alleles from nonpneumococcal species. Type A *ddl* alleles are commonly found among both PSSP and PNSP isolates.

PFGE types 6 and 7, found in single isolates of serotypes 6A and 19F, respectively, represent new PNSP strains that shared only four matching alleles with the closest seven-allele profiles in the MLST database. Two of the alleles obtained from the PFGE type 7 isolate, *gki57* and *ddl75*, were newly discovered in this isolate.

The single multiresistant serotype 23F, PFGE type 8 isolate shared allelic profile ST242 with the internationally disseminated multiresistant Taiwan^{23F}-15 clone (11).

The single multiresistant serotype 19F, PFGE type 9 isolate shared allelic profile ST88 with numerous recorded isolates of the "minor multiresistant Spanish serotype 19F clone" recovered in 1989 and 1997 (www.mlst.net).

One of the seven PFGE type 10, serotype 23F, antibiotic-susceptible isolates was found to share five alleles with three previously identified allelic profiles found among serotype 23F isolates recovered in the United States and Denmark (ST37, ST38, and ST39). All three profiles have been associated with antibiotic-sensitive isolates in Denmark, and ST37 has additionally been found in highly cephalosporin resistant isolates recovered in the United States, one of which is the type strain for clone Tennessee^{23F}-14 (11).

Finally, one of the seven erythromycin-resistant, PFGE type 11, serotype 14 isolates was found to share allelic profile ST15 with analogous erythromycin-resistant, invasive isolates recovered from the United Kingdom. PFGE type 11 is also seen in clone England^{14}-9 (11), with allelic profile ST9. ST9 differs at only one allele (*xpt*) from ST15.

PBP gene restriction profiles. With one exception, all isolates yielded PCR products for *pbp2b* and *pbp2x*. The negative *pbp2b* result is shown for the single non-penicillin-susceptible, PFGE type 9 isolate shown in Table 1 and is probably due to an altered primer annealing sequence(s). The overall data are consistent with the likelihood that all or the majority of the PNSP isolates described here carried mosaic *pbp2b* and *pbp2x* gene sequences that originated from past recombination events with nonpneumococcal species. The majority of isolates within each PFGE type displayed conserved PBP gene profiles.

Among the 22 PNSP isolates, nine distinct *pbp2b* restriction profiles were observed. Profile pbp2b-6 accounted for seven PFGE type 1 isolates for which penicillin MICs were 0.5 to 2 μg/ml. Profiles pbp2b-10, pbp2b-12, and pbp2b-5 were previously seen in invasive isolates recovered in the United States in 1997 for which penicillin MICs were less than 1.0 μg/ml (6) but greater than or equal to 0.12 μg/ml (unpublished data). *pbp2b* profiles 27 to 30 had not been previously documented and were seen among isolates for which penicillin MICs were 0.5 to 2 μg/ml. In contrast, only the previously described wild-type sensitive *pbp2b* profiles (2b-1 or 2b-2) (6) were seen among the 51 PSSP isolates.

Similar results were seen among *pbp2x* profiles, where 49 of the 51 PSSP isolates displayed the "sensitive" profile pbp2x-1 or pbp2x-8 (Table 1) (6). Two PSSP isolates showed profiles (pbp2x-26 and pbp2x-30) which we had not seen previously. Other than one intermediately penicillin resistant isolate (PFGE type 3.1) that exhibited the profile pbp2x-8, known *pbp2x* restriction profiles among PNSP isolates have been previously seen exclusively among isolates for which penicillin MICs were >1.0 μg/ml (pbp2x-2, pbp2x-9, and pbp2x-12) (6) or among isolates for which the penicillin MIC was 0.12 μg/ml (pbp2x-22 [unpublished data]). We had not previously seen PNSP isolates with *pbp2x* restriction profiles 27, 28, and 29.

It is possible that the set of seven PSSP type 23F isolates (PFGE type 10) with reduced susceptibility to ceftriaxone (MIC = 0.12 μg/ml) may contain wild-type "sensitive" *pbp2b* alleles and mosaic forms of *pbp1a* and *pbp2x* (15). Although profile pbp2x-8 has previously been seen only in beta-lactam-sensitive isolates, this profile could represent multiple alleles encoding both resistant and sensitive forms of Pbp2x. One of the seven isolates revealed pattern pbp2x-30, which we had not seen previously.

PspA sequence types. We compared PspA clade-defining region sequences from this isolate set to sequences of this vaccine candidate that had been previously obtained from internationally disseminated clones and common penicillin-resistant clones found in the United States (1, 7). PspA sequences were obtained from one representative PNSP isolate for each PFGE type and from one representative PSSP isolate for each of the most prevalent PFGE types. Additionally, *pspA* sequences were obtained from isolates of the same PFGE types that displayed divergent *pspA* restriction profiles.

Thirty-four *pspA* amplicon restriction profiles were obtained from the 73 isolates (Table 1) (1 isolate was PCR negative). As with PBP gene amplicon restriction profiles, the majority of isolates within each PFGE type shared identical *pspA* restriction profiles, although occasionally isolates with unrelated PFGE types shared identical *pspA* restriction profiles. It should be noted here that highly related *pspA* genes that share identical clade-defining regions often differ in their restriction profiles due to differing numbers of tandem proline-rich repeats that lie adjacent to the clade-defining region (1). It was interesting that the PSSP serotype 24F isolate (PN131) was shown to be highly related to France^{9V}-3 (Table 2) and additionally shared an identical PspA clade-defining region.

With one exception, all deduced clade-defining regions were

97 to 100% identical to previously described PspA clade-defining regions. Each of the common clades, clades 1 to 5, was represented by at least one isolate. Only one highly divergent PspA sequence was obtained from a serotype 8 isolate. This PspA clade-defining region sequence was only 93.5% identical to its closest match, a clade 4 PspA from the clone South Africa^{19A}-7 (1). In a recent survey of commonly occurring PNSP clones within the United States, we found no isolates with a clade 4 PspA sequence (1); however, no extensive survey among serotypes within which penicillin resistance rarely occurs has been published.

DISCUSSION

Penicillin-resistant pneumococcal isolates are recovered infrequently in Italy compared to neighboring European countries (13). In this paper, we present the genotypes of a selection of PNSP clones that cause invasive disease in this country. Although the number of available PNSP isolates was limited, these isolates are likely to represent a subset of common virulent pneumococcal clones in Italy. Only two different clonal types (PFGE types 1 and 8, corresponding to allelic profiles ST162 and ST242, respectively [Tables 1 and 2]) described in this study exhibited full penicillin resistance, and representatives of these two clones ((Taiwan^{23F}-15 and France^{9V}-3) were characterized in other countries years before this study. Full penicillin resistance requires multiple genetic alterations in multiple targets, and it appears that the majority of PNSP clones circulating in Italy have not yet accumulated a full complement of genetic changes that result in high-level β-lactam resistance.

Sets of antibiotic-resistant isolates highly related to 3 of the 16 internationally disseminated multiresistant clones described by the Pneumococcal Molecular Epidemiology Network (Taiwan^{23F}-15, France^{9V}-3, and England^{14}-9) (11) are described here, providing further evidence that antibiotic-resistant isolates expand clonally due to an evolutionary advantage. It will be of interest to track the occurrence of isolates with the allelic profiles Cdc41 and Cdc42 (temporary designations assigned before submission of strains to the MLST database). Although these two isolates belong to serotypes commonly observed among PNSP isolates, their allelic sequence types were quite divergent from the closest matches in the extensive pneumococcal MLST database (www.mlst.net).

Lack of susceptibility to penicillin among serotypes not previously associated with β-lactam resistance is reason for concern. The newly licensed 7-valent conjugate vaccine in the United States specifically targets serotypes commonly associated with penicillin resistance that infect infants and young children. In this study, we present clear circumstantial evidence of PNSP clonal sets where horizontal transfer events have resulted in expression of a new capsular serotype. In the MLST database and in our study, allelic profile ST63 was found among intermediately penicillin resistant type 15A isolates, which are rarely associated with full resistance (Table 2). It is possible that the PNSP ST63, serotype 19A isolates represent the original serotype associated with ST63, since serotype 19A strains are commonly not susceptible to penicillin. Similarly, we believe it is likely that a PNSP serotype 14, ST230 strain was genetically transformed to express serotype 24F (Table 2). With some of the pairs shown in Table 2, it is difficult to envision the chain of genetic events that have transpired. For example, the closest match to allelic profile Cdc40, in an unusual PNSP serotype 35F strain, is ST136 from a serotype 6B strain for which the penicillin MIC is the same. ST136 is characterized by a highly divergent type B *ddl* allele, presumably as a result of hitchhiking during a transformation event where donor DNA was from a nonpneumococcal species (5). Allelic profile Cdc40 has a type A *ddl* allele commonly found among pneumococcal strains. Thus, Cdc40 and ST136 exhibit the same level of penicillin resistance yet show evidence of different transformation events at the *pbp2b* chromosomal region.

Twenty of the 22 PNSP isolates were susceptible to ceftriaxone by tests conducted according to the NCCLS guidelines. However, pneumococcal isolates for which extended-spectrum cephalosporin MICs are ≥0.25 μg/ml generally have mosaic PBP genes (6). In this study we found that nearly all isolates for which ceftriaxone MICs were ≥0.25 μg/ml had PBP gene amplicon profiles not shared with isolates for which MICs of this antibiotic were lower. These isolates have started the process of accumulating multiple mutations that are necessary for the expression of full resistance to these antibiotics.

Even though the surface PspA protein displays a remarkable degree of variation among pneumococcal strains, it is a promising vaccine candidate (7), and in addition the *pspA* locus provides a useful molecular marker. In this study, we found that the *pspA* sequence types associated with PNSP isolates in Italy varied little from those of antibiotic-resistant clones circulating within the United States. We also found that the majority of isolates within a given genetic set showed identical *pspA* amplicon restriction profiles. We are hesitant to conclude that PFGE type 15 represents a clone with both serotype 4 and 18C isolates, since we have not performed MLST on these isolates and one can on rare occasions find fortuitous similarity between PFGE profiles that gives a false indication of relatedness (B. Beall, unpublished data). However, the finding of *pspA* amplicon restriction profiles conserved between these isolates does provide further circumstantial evidence that these serotype 4 and 18C isolates are in fact closely genetically related.

In conclusion, this study describes new PNSP clones and evidence of transformation events resulting in unusual serotype-clone type associations. These studies have been greatly assisted by the accessibile MLST database (www.mlst.net) and the Pneumococcal Molecular Epidemiology Network (11), which have international scope in tracking clones of virulent pneumococci.

ACKNOWLEDGMENTS

We thank Marina De Cesaris, Manola Valente, and Federica Bonanni at the University "Campus Biomedico" and Zhongya Li and Varja Sakota at the Centers foe Disease Control and Prevention (CDC) for assistance with PFGE, PCR, and sequencing.

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