Phenotypic and Genotypic Characterization of Two Penicillin-Susceptible Serotype 6B *Streptococcus pneumoniae* Clones Circulating in Italy

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Twenty-nine penicillin-susceptible serotype 6B strains isolated from patients with invasive diseases and from healthy carriers were examined by different genotyping methods. Ten groups were identified on the basis of the pulsed-field gel electrophoresis (PFGE) profiles, and two of these contained multiple isolates and were analyzed further. PFGE group 1 comprised 12 isolates, the majority of which had a multiresistant phenotype (resistance to erythromycin, clindamycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole), corresponding to that of a clone previously described in the Mediterranean area and related to penicillin-resistant clone Spain^{6B}-2. The *pbp2b*, *pbp2x*, *dhf*, and *pspA* genes of the isolates had identical restriction profiles; and the partial sequence of *pspA* was identical to that of clone Spain^{6B}-2. In all isolates the resistance determinants *erm*(B) and *tet*(M) were inserted in a Tn1545-like element; 11 isolates carried *cat* as part of the integrated plasmid pC194. Multilocus sequence typing (MLST) performed with two isolates confirmed that their profiles corresponded to that of the Mediterranean clone. PFGE group 2 comprised nine strains, of which the majority showed no antibiotic resistance. Their *pspA* profiles were different, and the partial sequences obtained for two representative isolates indicated the presence of PspA proteins of different clades. The MLST profile of one strain was identical to that of a serotype 6B strain from the United Kingdom, while two other isolates were novel one-allele variants. This clone appears to be related (five of seven identical alleles) to two other internationally disseminated clones, Hungary^{19A}-6 and Poland^{23F}-16, both of which are penicillin resistant. The presence of antibiotic-susceptible isolates of this clone suggests that traits other than antibiotic resistance can make a clone successful.

In the last decade, the rate of antibiotic resistance among *Streptococcus pneumoniae* strains has increased worldwide and antibiotic resistance has become a global problem. Rates of resistance vary markedly in different geographic areas and also between neighboring countries. In Italy, the rate of penicillin resistance is moderate, ranging from 9 to 13% from 1999 to 2001, which represents the lowest rate among the countries in southern Europe (8). On the contrary, the rate of resistance to macrolides is very high and has increased steadily in the recent years, reaching approximately 30% among invasive isolates (8, 23) and over 50% among isolates from young carriers (24). The majority of the resistant strains carry the *erm*(B) gene, while only approximately 20% carry *mef*(A) (5), which is different from the case for the erythromycin-resistant strains isolated in North America (9). The *erm*(B) gene is associated with the macrolide-lincosamide-streptogramin B resistance phenotype (the MLS_B phenotype) in *S. pneumoniae*, characterized by high levels of resistance to erythromycin and the other macrolides and to clindamycin (13). In Italy, the majority of strains with the MLS_B phenotype are also resistant to tetracycline, and

some of them are resistant to chloramphenicol (23); therefore, they can be considered multidrug resistant (MDR), although they generally retain susceptibility to penicillin.

In recent years, molecular methods have enabled investigators to identify penicillin-resistant or MDR clones that are particularly successful in spreading and becoming established in distant geographic areas (7, 18, 20). In a recent study of invasive isolates from Italian patients, we found antibiotic-resistant strains highly related to internationally disseminated resistant clones (6).

In 2001, Syrogiannopoulos and coworkers (31) described serotype 6B strains colonizing healthy children in Greece which were MDR (resistant to erythromycin, clindamycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole) but penicillin susceptible. Isolates with similar characteristics were found in young carriers in other countries, such as Italy and Israel (32). By molecular methods, the isolates were found to belong to the same clone, designated the Mediterranean clone, which is related to one of the most widespread antibiotic-resistant clones, the Spanish/Icelandic clone, or clone Spain^{6B}-2 (18). Clone Spain^{6B}-2 isolates are typically resistant to several antibiotics, including penicillin; however, some isolates from Iceland have lost some of the resistance determinants (34).

Serogroup 6, represented mainly by serotype 6B, ranks fourth among the different capsular groups or types among invasive isolates from Italian patients and is the second most

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common capsular type among isolates from Italian children ages 0 to 5 years. Over 40% of serogroup 6 isolates from Italy are erythromycin resistant (A. Pantosti, D. Boccia, F. D'Ambrosio, S. Recchia, G. Orefici, M. L. Moro, et al., submitted for publication).

This study describes the genotypic characterization of two penicillin-susceptible serotype 6B clones circulating in Italy, one of which corresponds to the MDR Mediterranean clone. The two clones have been characterized for their genetic determinants of resistance and for the allele coding for PspA, an antigenically variable surface protein which is considered a potential pneumococcal vaccine candidate (2).

MATERIALS AND METHODS

Sources of strains. Twenty-nine capsular type 6B strains of *S. pneumoniae* were examined. Nineteen of these were from blood or cerebrospinal fluid of patients with invasive pneumococcal diseases and were isolated in different areas of Italy from 1997 to 2000 (19, 23). The other 10 isolates were from healthy children attending day-care centers in Rome (24). Ten isolates (eight from patients with invasive disease and two from carriers) exhibited a resistance phenotype compatible with that of the Mediterranean clone (susceptibility to penicillin and resistance to erythromycin, clindamycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole) (31). The additional 19 capsular type 6B isolates displayed different resistance patterns or were susceptible to all the antibiotics tested.

Antibiotic susceptibility testing. Susceptibilities to penicillin, erythromycin, clindamycin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole were assayed by the Etest (AB Biodisk, Solna, Sweden), according to the recommendations of the manufacturer. Categories were defined by using the breakpoints established by the NCCLS (21). Susceptibility to kanamycin was assessed by a disk diffusion method with 6-mm paper disks impregnated with 500 µg of kanamycin and prepared in-house (29).

Detection of resistance determinants and transposon markers. Pneumococcal isolates were examined by PCR for the presence of the resistance determinants *erm*(B), *mef*(A), *tet*(M), *cat*, and *aphA-3*. A duplex PCR was used to amplify the genes *erm*(B) and *mef*(A) with primer pairs EB1-EB2 and ME1-ME2, as described previously (22). Internal fragments of the *tet*(M) and *cat* genes were amplified with primer pairs TETMd-TETMr and CATd-CATr, respectively, described by Marchese et al. (15). The *aphA-3* gene, which confers resistance to kanamycin, was detected by amplification of a 292-bp fragment by using the oligonucleotide primer pairs described by van Asselt and coworkers (33).

The isolates were examined for the presence of the *int* gene, which codes for one of the two proteins involved in the excision of the Tn916-Tn1545 family of conjugative transposon (4), by using INTf (5'-GACTGGAGAGACCAACGA A-3') and INTTr (5'-CATCATGCCGTTGTAATCAC-3') (GenBank accession no. X61025) (25). To verify the association of the *cat* gene with plasmid pC194, which has been found to be integrated in the chromosome of *S. pneumoniae* (35), a 320-bp fragment of the *rep* gene of pC194 (*rep*_{pC194}) was amplified with oligonucleotide probes REPf (5'-GGGTTTATCTTTGATGATGC-3') and REPr (5'-CACGCCATAATCTAACCAT-3') (GenBank accession no. V01277) (12).

All PCRs were performed in a 50-µl volume with boiled pneumococcal bacterial cells as the template in a personal Mastercycler (Eppendorf AG, Hamburg, Germany).

Extended PCR to investigate the association of *erm*(B) and *tet*(M). To determine whether the *erm*(B) and *tet*(M) genes were carried by the same genetic element and, therefore, whether they were in close proximity to each other in the chromosome, amplification of the DNA comprising *erm*(B) and *tet*(M) was attempted. The four possible combinations of primer pairs associated with the possible reciprocal orientation of the genes were tested by using oligonucleotides EB1, EB2, TETMd, and TETMr, described above. The reactions were performed in a 50-µl volume; the mixture contained 2 mM MgCl₂, 200 µM each deoxyribonucleoside triphosphate, 0.5 µM each oligonucleotide, and 0.5 U of DNA polymerase (*TaKaRa Ex Taq*; Takara Shuzo Co., Otsu, Japan). The template was 1 µl of purified pneumococcal chromosomal DNA, obtained from a commercial kit (DNA Midi kit; Qiagen SpA, Milan, Italy). The cycling parameters were 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 52°C, and 8 min at 72°C.

PCR-restriction fragment length polymorphism (RFLP) analysis and se-

quencing. Internal fragments of *pbp2b* and *pbp2x* (the genes coding for penicillin binding proteins), *dhf* (the gene conferring resistance to trimethoprim), and *pspA* (the gene coding for the surface protein PspA) were obtained by PCR amplification, digested with restriction enzymes, and analyzed as described previously (2, 10, 30). The sequences of the *dhf* gene and of a 700-bp fragment of *pspA*, encompassing the clade-defining region and the proline-rich region (2, 10) were obtained for selected isolates.

PFGE and cluster analysis. *Sma*I macrorestriction typing was performed as described previously (10). Isolates with identical pulsed-field gel electrophoresis (PFGE) patterns were assigned to the same PFGE type and subtype. Isolates differing by one to six bands were considered genetically related and were assigned to the same PFGE type but to different subtypes. Isolates differing by more than six bands were classified as different PFGE types. PFGE types were analyzed with Bionumerics software for Windows (version 2.5; Applied Maths, Ghent, Belgium). The DNA banding patterns were normalized with bacteriophage lambda concatemer ladder standards. Comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages and with the Dice similarity coefficient. A tolerance of 1.5% in band position was applied during comparison of the DNA patterns.

MLST. Five strains were examined by multilocus sequence typing (MLST) by the method recommended at the MLST website (http://www.mlst.net/new/databases/_s_pneumoniae.htm) and were compared with the strains in the MLST database.

RESULTS

Clustering by PFGE analysis. Twenty-nine penicillin-susceptible serotype 6B pneumococcal isolates were genotyped in order to establish their genetic relatedness and to compare them with the pneumococcal lineages of serotype 6B described in other countries. As depicted in Table 1 and Fig. 1, 10 different PFGE types were found. Two PFGE groups contained multiple isolates: PFGE type 1 comprised 12 isolates and consisted of six different PFGE subtypes (subtypes 1.1 to 1.6), and PFGE type 2 comprised 9 isolates and consisted of seven different PFGE subtypes (subtypes 2.1 to 2.7). Eight PFGE types were represented by single isolates.

The profiles of the PFGE type 1 isolates closely resembled the profile of reference clone Spain^{6B}-2 (18), differing by 3 or fewer bands among approximately 15 clearly visible bands. The 10 isolates that were included in the study because of their multiresistance profile, which corresponded to that described for the Mediterranean clone, were PFGE type 1. In addition, this group included two isolates whose resistance patterns differed slightly from that of the Mediterranean clone: one isolate was susceptible to tetracycline, and the other was susceptible to chloramphenicol and trimethoprim-sulfamethoxazole (Table 1).

PFGE type 2 included three isolates that were resistant to erythromycin, clindamycin, and tetracycline and six isolates that were susceptible to all the antibiotics tested. The single strains of the remaining eight PFGE types showed different susceptibility profiles (Table 1). Further genotypic characterization was performed with the strains belonging to PFGE types 1 and 2.

Genotyping by *pbp2b*, *pbp2x*, *dhf*, and *pspA* profiling and sequencing. The *pbp2b* and *pbp2x* restriction profiles obtained for PFGE type 1 and type 2 isolates were identical and corresponded to the profiles previously found for isolates fully susceptible to β-lactam antibiotics (6, 10).

The *dhf* restriction profiles of isolates of both PFGE type 1 and type 2 were also identical and were *dhf*-1. However, most isolates of PFGE type 1 were resistant to trimethoprim-sulfamethoxazole, while isolates of PFGE group 2 were susceptible. The *dhf*-1 profile was previously found to be associated mainly

FIG. 1. Genetic relatedness of the 29 serotype 6B *S. pneumoniae* strains studied. Comparison of the banding patterns was performed by the unweighted pair group method with arithmetic averages by using the Dice similarity coefficient. The strain codes for representative isolates, the PFGE subtypes, and a dendrogram with percentages of similarity are shown. The numbers of isolates with an identical PFGE subtype are indicated in parentheses.

with trimethoprim susceptibility (10). To find the genetic basis for the phenotypic resistance to trimethoprim-sulfamethoxazole, the partial sequence of the *dhf* gene of a representative PFGE group 1 isolate (isolate PN68) was obtained. Eleven amino acid substitutions compared with the wild-type *dhf* gene sequence were observed, including the critical substitution necessary for trimethoprim resistance at residue 100 of dihydrofolate reductase (Ile-100→Leu) (16). Most of the amino acid changes observed in the sequence from PN68 have already been found in other trimethoprim-resistant *S. pneumoniae* strains described in previous studies (10, 16), with the exception of a novel substitution at position 143 (Val-143→Ile), which had not been observed previously.

pspA gene restriction analysis of the PFGE type 1 isolates yielded only a single RFLP type, and this type was identical to type k1 found previously in the Spain^{6B}-2 reference clone (2). Accordingly, the sequence of a fragment of *pspA*, encompassing the clade-defining region and the proline-rich region, from a representative PFGE type 1 isolate (isolate PN68) was identical to that of reference clone Spain^{6B}-2 (GenBank accession no. AF254257) (2), indicating a PspA sequence of clade 1. *pspA* restriction analysis of PFGE type 2 isolates yielded two different restriction types not found in previous studies, designated *pspA*-35 and *pspA*-36, respectively. Three isolates with an identical PFGE subtype (subtype 2.4) did not yield any amplification product for *pspA* by PCR. This negative result is probably due to an altered annealing sequence at the primer site.

The partial *pspA* sequence of an isolate yielding restriction profile *pspA*-35 (isolate PN102) was 99% identical at the deduced amino acid level to the sequence of reference strain 127 (GenBank accession no. AF255544) (2), a serotype 19F strain. This sequence corresponds to a clade 3 PspA protein. The partial sequence of an isolate with RFLP profile *pspA*-36 (isolate 1404) was found to be identical at the amino acid level to that of a serotype 5 isolate, DBL5 (GenBank accession no. AF071810), whose PspA protein belongs to clade 2 (11).

Characterization of the resistance determinants. All isolates belonging to PFGE type 1 were positive for the *erm*(B) gene by PCR, while *mef*(A) was not detected. This is consistent with the resistance phenotypes of the isolates, which showed high levels of resistance (MICs ≥ 256 μ g/ml) to erythromycin and clindamycin (13). All isolates were positive for the *tet*(M) gene, including isolate PN65, which was tetracycline susceptible. To verify the hypothesis that *erm*(B) and *tet*(M) reside in a transposable element related to Tn1545, the isolates were tested for the presence of the kanamycin resistance gene, *aphA*-3, which is present in Tn1545 (3), and the integrase gene, *int*, which is characteristic of the Tn916-Tn1545 family of conjugative transposons (4). For all the isolates, PCR for *aphA*-3 was negative (Table 1), which is in accordance with the susceptibilities of the isolates to high levels of kanamycin; however, PCR for the *int* gene was positive. The proximities of *erm*(B) and *tet*(M) were investigated in four isolates representative of different PFGE subtypes or antibiotypes (isolates PN68, PN65, 9111, and

TABLE 1. Genetic characterization of serotype 6B isolates

PFGE type.subtype	Isolate(s)	Antibiotype ^a	Presence of the following resistance determinants and transposon markers:							Type by PCR-RFLP analysis	
			<i>erm</i> (B)	<i>tet</i> (M)	<i>cat</i>	<i>aphA</i> -3	<i>int</i>	<i>rep</i> _{PC194}	<i>erm-tet</i> _{Tn1545} ^b	<i>pbp2b/2x/dhf</i>	<i>pspA</i>
1.1	SP036, SP065, PN68	EM, CC, TC, CH, SXT	+	+	+	—	+	+	+ ^c	1/1/1	k1
1.1	PN65	EM, CC, CH, SXT	+	+	+	—	+	+	+	1/1/1	k1
1.2	9111	EM, CC, TC, CH, SXT	+	+	+	—	+	+	+	1/1/1	k1
1.3, 1.4, 1.5	PN120, SP014, SP245, PNS12, PNS17, 1211	EM, CC, TC, CH, SXT	+	+	+	—	+	+		1/1/1	k1
1.6	PN20	EM, CC, TC	+	+	—	—	+	—	+	1/1/1	k1
2.1	161	EM, CC, TC	+	+	—	—	+	—	+	1/1/1	35
2.2	225	EM, CC, TC	+	+	—	—	—	—	— ^d	1/1/1	35
2.3	PN102	No resistance	—	—	—	—	—	—		1/1/1	35
2.4	PN6, 165, 531	No resistance	—	—	—	—	—	—		1/1/1	Neg ^e
2.5	1404	EM, CC, TC	+	+	—	—	+	—	+	1/1/1	36
2.6, 2.7	SP353, SP350	No resistance	—	—	—	—	—	—		1/1/1	36
3.1	2210	SXT								ND ^f	ND
4.1	5310	SXT								ND	ND
5.1	1114	EM, CC, TC, SXT								ND	ND
6.1	PN64	EM, CC, SXT								ND	ND
7.1	SP099	TC, SXT								ND	ND
8.1	SP085	No resistance								ND	ND
9.1	SP147	SXT								ND	ND
10.1	SP218	No resistance								ND	ND

^a Resistance to the indicated antibiotics. EM, erythromycin; CC, clindamycin; TC, tetracycline; CH, chloramphenicol; SXT, trimethoprim-sulfamethoxazole.

^b Detection of *erm*(B) and *tet*(M) within an element similar to Tn1545.

^c One representative isolate (PN68) was examined.

^d PCR was positive with different primers, indicating the presence of a different element (see text).

^e Neg, no amplification.

^f ND, not determined.

PN20) (Table 1) by an extended PCR approach. The combination of oligonucleotide primers EB1 and TETMr produced an amplicon of approximately 12 kb, indicating that *erm*(B) and *tet*(M) are adjacent in the chromosome and have the same orientation. The distance between the two genes and their reciprocal orientations are compatible with their location on a Tn1545-like transposon (3). PCR assays for *cat* and *rep*_{PC194} were positive for all isolates of this group except one, isolate PN20, which was chloramphenicol susceptible. This indicates that resistance to chloramphenicol in the resistant isolates could be associated with an integrated form of plasmid pC194 (35). Among PFGE type 2 isolates, the *erm*(B) and *tet*(M) genes were detected in only three isolates, which were resistant to erythromycin and tetracycline. In these isolates PCR for *int* was also positive. The extended PCR with primer pair EB1-TETMr was positive for two of these isolates (isolates 161 and 1404), suggesting that the organization of the element carrying *erm*(B) and *tet*(M) is similar to that present in PFGE type 1 isolates. The extended PCR with primer pair EB1-TETMr was negative for resistant isolate 225. However, a PCR product of approximately 3,000 bp was obtained with primer pair EB2-TETMd. The distance between the two genes and their reciprocal positions are compatible with the structure of the composite transposon Tn3872 (17).

No isolate belonging to the PFGE type 2 group of strains was resistant to chloramphenicol, and accordingly, *cat* and *rep*_{PC194} were not detected.

MLST. MLST was performed with two isolates of PFGE type 1 (isolates PN68 and PN20) and three representative isolates of PFGE type 2 (isolates PN102, PN6, and 1404) showing different patterns of antibiotic resistance and different *pspA* restriction profiles. The allelic profiles were compared with

those available at the MLST website (http://www.mlst.net/new/databases/s_pneumoniae.htm). The results are summarized in Table 2.

The sequence types (STs) of PN68 and PN20, which lacked resistance to chloramphenicol and trimethoprim-sulfamethoxazole, were identical to the ST of an isolate from Greece, representative of the Mediterranean clone (ST273). The profile differed from that of clone Spain^{6B}-2 at two alleles (*xpt* and *ddl*) and from that of its variant from Finland at only one allele (*ddl*). These particular clones from Spain and Finland are penicillin resistant (18).

Three PFGE group 2 isolates (isolates PN6, PN102, and 1404) were submitted for MLST analysis. Strain PN6 yielded ST176, shared with an invasive isolate from the United Kingdom and with a single-locus variant from Denmark, both of which are penicillin susceptible and belong to capsular type 6B. The allelic profiles of the other two isolates showed that both were of new STs, with the STs being single-locus variants of ST176. The ST of strain 1404 differed from ST176 at the *spi* allele, while strain PN102 exhibited a novel sequence in the *gdh* allele. The MLST database contains 58 isolates, if the Italian isolates examined in this study are excluded, with at least five of seven alleles matching the ST176 allelic profile. Of these isolates, the large majority (48 isolates) are from northern Europe, and none are from Greece, Spain, or France. Although the majority of these 58 isolates are serotype 6B and penicillin susceptible, a few are of different serotypes (serotypes 23F, 19A, and 3) and are penicillin resistant (http://www.mlst.net/new/databases/s_pneumoniae.htm). Among these are the penicillin-resistant international clones Hungary^{19A}-6 and Poland^{23F}-16 (Table 2).

TABLE 2. Properties and MLST types of Italian 6B isolates belonging to PFGE types 1 and 2 and comparison with isolates in the MLST database

Isolate	Source ^a	Country	Serotype	Antibiotyp ^b	ST	Allelic profile ^c						
						<i>aroE</i>	<i>gdh</i>	<i>gki</i>	<i>recP</i>	<i>spi</i>	<i>xpt</i>	<i>ddl</i>
PN68	CSF	Italy	6B	EM, CC, TC, CH, SXT	273	5	6	1	2	6	1	14
PN20	CSF	Italy	6B	EM, CC, TC	273	5	6	1	2	6	1	14
JJ270-6B	Nasopharynx	Greece	6B	EM, TC, CH	273	5	6	1	2	6	1	14
87-027157-6B	NR	Finland	6B	PEN, EM, TC	238	5	6	1	2	6	1	28
Finland ^{6B} -12	NR	Finland	6B	PEN, EM, CC, TC, SXT	270	5	6	43	2	6	1	28
Spain ^{6B} -2	NR	Spain	6B	PEN, TC, CH, SXT	90	5	6	1	2	6	3	4
PN6	CSF	Italy	6B	No resistance	176	7	13	8	6	10	6	14
PN102	CSF	Italy	6B	No resistance	560	7	63	8	6	10	6	14
1404	Nasopharynx	Italy	6B	EM, TC	559	7	13	8	6	5	6	14
M49-6B	CSF	United Kingdom	6B	No resistance to PEN or EM	176	7	13	8	6	10	6	14
Hungary ^{19A} -6	NR	Hungary	19A	PEN, EM, CC, TC, CH,	268	7	13	42	6	10	6	56
Poland ^{23F} -16	NR	Poland	23F	PEN, EM, CC, TC, CH, SXT	173	7	13	8	1	10	6	36

^a CSF, cerebrospinal fluid; NR, not reported.

^b Resistance to the indicated antibiotics. The antibiotypes of isolates not examined in this study are derived from a previous report (18) and the MLST website (http://www.mlst.net/new/databases/s_pneumoniae.htm). EM, erythromycin; CC, clindamycin; TC, tetracycline; CH, chloramphenicol; SXT, trimethoprim-sulfamethoxazole.

^c In each group, variant alleles are in boldface.

DISCUSSION

In the past decade, molecular methods for recognition of genetic relatedness in *S. pneumoniae* have been applied mainly to penicillin-resistant isolates; hence, the view is that the emergence of successful pneumococcal clones is linked to the evolutionary advantage conferred by antibiotic resistance.

In the present study we examined strains from Italy for the presence of the MDR penicillin-susceptible serotype 6B clone described in Greece, using recent molecular typing techniques. Strains with an antibiotic resistance phenotype corresponding to that of the Greek MDR clone had previously been isolated from young carriers in central Italy (27) and from individuals of all ages with invasive diseases (23).

By means of PFGE genotyping, we demonstrated the circulation in Italy of several different lineages of penicillin-susceptible serotype 6B strains, among which two groups of strains, representing two distinct clones, were recognized. Each included isolates that colonized healthy carriers and isolates from patients with invasive diseases. By MLST we could prove that one clone in fact corresponded to the Greek MDR clone disseminated in the Mediterranean area. The other clone, which comprised a majority of susceptible isolates, was not related to the first clone, as it shared only one of seven alleles (the *ddl* allele) with the Greek MDR clone.

The Italian isolates of the Greek MDR clone appeared to have very homogeneous genetic traits, with few exceptions. They shared the same resistance determinants and elements and the same PCR-RFLP profile for *pspA*. The MLST allelic profiles were determined for two of between these strains and were found to be identical the strains and to that of the representative Greek MDR serotype 6B isolate deposited in the MLST database (http://www.mlst.net/new/databases/s_pneumoniae.htm). Interestingly, the partial deduced PspA sequence, obtained for one isolate, corresponded to that of the Spain^{6B}-2 clone (2), providing further evidence that the MDR clone is a close relative of clone Spain^{6B}-2 (31). Genotyping by *pspA* RFLP analysis, a method originally proposed by Swiatlo et al. (30) to characterize serotype 9L isolates,

has proved very useful for the monitoring of clonal relationships. Whether the MDR clone is the ancestor of the Spain^{6B}-2 clone or is a descendant that has resumed susceptibility to penicillin remains to be ascertained. Since pneumococcal isolates with antibiotic resistance patterns similar to that of the Greek MDR clone were very rare in Italy in the first half of the past decade (14), this clone has probably arisen in another country and has spread to Italy only recently.

In a recent study on the global evolution of serogroup 6 pneumococci, based on the allelic profile of a different combination of seven housekeeping genes, the Spain^{6B}-2 clone and the MDR Greek clone have identical allelic profiles and are therefore considered to be the same clone. This clone appears to have evolved from a tetracycline-resistant strain isolated in Uruguay (26).

Besides showing identity to isolates deposited in the MLST database, a recent paper describes serotype 6B isolates with the same allelic profile as the MDR Greek clone, but with different resistance patterns (28). These isolates were from Iceland or Portugal and generally lacked resistance to chloramphenicol and trimethoprim-sulfamethoxazole. Moreover, single-allele variants of the MDR Greek clone were isolated in Alaska. It is thus apparent that the MDR clone is not restricted to the Mediterranean area but has disseminated in several countries and different continents, and therefore, it deserves to be considered a principal international antibiotic-resistant clone. The characteristic resistance to multiple antibiotics in this clone appears to be due to the presence of a Tn1545-like transposon carrying erythromycin and tetracycline resistance determinants and to another element, comprising the integrated plasmid pC194, which carries the chloramphenicol resistance determinant *cat*. Although we have not studied the linkage between the Tn1545-like element and the element carrying *cat* in the pneumococcal chromosome, we can hypothesize that the MDR clone contains a novel composite transposon [$\Omega(tet\ erm\ cat)$], similar to Tn5253 (1), with the addition of the erythromycin resistance gene *erm*(B).

The genetic traits of the PFGE type 2 clone are more heterogeneous than those of the PFGE type 1 clone. The isolates of the PFGE type 2 clone have different resistance phenotypes and genotypes and showed at least two variants of the *pspA* gene belonging to different clades. The MLST allelic profiles of the isolates examined were similar but not identical, differing at a single locus. Close relatives of this clone, which differed at two of seven alleles, include a variety of strains isolated in northern Europe and also two internationally disseminated penicillin-resistant clones, Hungary^{19A}-6 and Poland^{23F}-16. These northern European variants have not yet been detected in southern Europe. In two isolates of the PFGE type 2 clone, erythromycin and tetracycline resistance determinants appear to reside in a genetic element similar to that present in the PFGE type 1 clone. However, in a third resistant isolate of PFGE type 2, a different element was found, possibly resembling the composite transposon Tn3872, described in resistant strains of this serotype (17).

The genetic homogeneity of isolates of the MDR PFGE type 1 clone suggests a recent origin for this clone, whose diffusion has probably been favored by the use of antibiotics. At variance, PFGE group 2 isolates, which also include antibiotic-susceptible strains, show dissimilarity at several hypervariable loci, probably as a result of recombination exchanges between the isolates of this clone and different pneumococcal strains. This suggests that the origin of this clone is not recent and that factors other than antibiotic resistance might have favored its diffusion.

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