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Properties of Novel International Drug-Resistant Pneumococcal Clones Identified in Day-Care Centers of Lisbon, Portugal

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In this study, 61 drug-resistant *Streptococcus pneumoniae* strains were characterized by multilocus sequence typing (MLST). These strains were representatives of 26 major clones (defined using pulsed-field gel electrophoresis) accounting for 93% of the 1,285 drug-resistant *Streptococcus pneumoniae* isolates recovered from the nasopharynges of healthy children attending day-care centers in Lisbon during 2001 to 2003. Using MLST, 13 of the 26 clones were found to be identical or closely related to 11 Pneumococcal Molecular Epidemiology Network (PMEN) clones, 4 clones were found to be unique as there were no identical or highly related allelic profiles deposited in the MLST database, and the remaining 9 clones had sequence types that matched or differed at a single or double locus from allelic profiles available in the MLST database. These nine clones were of serotypes 33F, 10A, 19A, 19F, 6A, 20, 24F, and 3, one was nontypeable, and, by MLST, they were found to be identical or highly related to isolates from disease origin that were dispersed internationally. Since the majority of these clones had serotypes that are not included in the 7-valent conjugate pneumococcal vaccine, monitoring of these clones is important for surveying their possible spread in the future. We propose the inclusion of these novel international clones in the PMEN.

Streptococcus pneumoniae (pneumococcus) is a major cause of invasive diseases, such as bacteremia, septicemia, and meningitis, and also causes less severe conditions, such as middle-ear infections, sinusitis, and recurrent bronchitis (48). It causes annually over one million deaths worldwide, mainly in children younger than 5 years of age, and the highest incidence is registered in developing countries (48).

The normal habitat of pneumococci is the nasopharynx, and pneumococcal nasopharyngeal colonization is the starting point for pathogenesis and horizontal spread of strains. The highest colonization rates are found in populations attending crowded places such as day-care centers. Children are frequently colonized by pneumococcus without having disease symptoms, as the mucosal membranes often confer immunity (15). However, when the host condition is altered due to internal and/or external factors, the existent balance is disturbed and disease may occur (15, 25).

For these reasons, it is important to enhance the knowledge of the dynamics of the colonization process (3), including surveillance and characterization of the nasopharyngeal pneumococci.

The continuous emergence of pneumococcal strains resistant to antimicrobial agents is a major cause of concern, narrowing the therapeutic alternatives for pneumococcal disease treatment. Respiratory tract infections, often caused by pneumococci, are the most common reason for antibiotic prescription in children (20). The emergence of penicillin-resistant pneumococci has led to the use of alternative antimicrobials, and macrolides in particular provide a good alternative for treatment of respiratory tract infections (21, 22). However, in the last decade, the prevalence of both penicillin- and macrolide-resistant pneumococci has rapidly increased worldwide (1, 9, 24, 42).

Although only a few drug-resistant pneumococcus (DRPn) clones have successfully achieved worldwide spread (23), with the introduction of the conjugate pneumococcal vaccine the emergence of novel pandemic drug-resistant clones expressing capsular serotypes not covered by the vaccine is a strong possibility.

The prevalence of DRPn clones may vary according to geographic area and also over time. Therefore, surveillance is important for detecting local epidemics, monitoring patterns of resistant bacteria, and helping in the choice of appropriate antimicrobial agents (35).

Typing techniques such as antimicrobial susceptibility testing, serotyping, and pulsed-field gel electrophoresis (PFGE) are commonly used for local surveillance of pathogens as pneumococci. For global epidemiology, multilocus sequence typing (MLST) (10) has become the most valuable tool (16).

In this study 61 DRPn strains were characterized by MLST in order to identify and relate their genetic backgrounds with the ones of isolates recovered in other geographic areas. These strains were representatives of the 26 most frequent clonal types that, together, accounted for 93% of the DRPn isolates

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recovered from the nasopharynges of healthy children attending day-care centers in the Lisbon and Oeiras regions during 2001 to 2003.

MATERIALS AND METHODS

Pneumococcal carriage isolates. From 2001 to 2003, 3,539 pneumococcal isolates were collected from the nasopharynges of healthy children (aged 6 months to 6 years) attending 13 day-care centers in Lisbon and Oeiras. These strains were collected during the European Resistance Intervention Study Reducing Resistance in Respiratory Tract Pathogens in Children project.

Antimicrobial susceptibility testing, according to the NCCLS guidelines (26) identified 1,285 DRPn. Oxacillin, clindamycin, erythromycin, tetracycline, chloramphenicol, levofloxacin, and sulfamethoxazole-trimethoprim were tested by the disk diffusion method. Penicillin and ceftriaxone MICs were determined with the Etest (AB Biodisk, Solna, Sweden) according to the manufacturers' instructions. Serotyping (43) and PFGE (38) were performed for all DRPn, and 80 clonal types were defined based on PFGE typing.

From this collection 61 pneumococci were selected for MLST according to the following criteria: two representative strains of clonal types with more than five isolates. These strains had the most common PFGE subtypes within the clone group. In addition, representatives of possible capsular switch events (strains sharing the same PFGE clonal type but with different capsular types) were also selected.

Pneumococcal reference strains. The 26 strains currently accepted by the Pneumococcal Molecular Epidemiology Network (PMEN) were used as reference strains (23; http://www.sph.emory.edu/PMEN/).

PFGE clonal types. Clonal types were defined using PFGE (7). Briefly, PFGE patterns were analyzed with Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium), and relatedness among the PFGE profiles was evaluated, using *S. pneumoniae* strain R6 as a molecular marker.

The dendrogram was generated from a similarity matrix calculated with the Jaccard coefficient, and patterns were clustered by the unweighted pair group method with arithmetic mean, using an optimization of 0% and a tolerance of 1.4%. By comparing the clusters generated by Bionumerics with the clusters obtained by visual classification using Tenover's criteria (44), a similarity of 60% was found to be the most appropriate cutoff above which isolates would belong to a common clone.

PFGE patterns obtained in this study were compared with those obtained for strains isolated between 1996 and 1999 in the same settings to determine their prevalence through time (27, 36–38) and with PFGE patterns of the 26 reference strains from PMEN.

The nomenclature of the PFGE patterns was given arbitrarily and is the same used in previous studies. Two or three identical capital letters were assigned to patterns found during 1996 to 1998. Two different capital letters were assigned to new PFGE profiles that were found since 1999.

MLST. MLST was performed as described previously (10). The primers used for PCR amplification were the ones originally described (10), with the exception of two primers: rec2-dn, GTT CCA TTT TCA ACC AAG GC, and spi2-up, AGA GTG GGG ATT ATT CCT CC. These two new primers were used to overcome difficult PCR amplifications observed for some isolates which DNA did not anneal adequately with the original primers, rec-dn and spi-up. Sequencing was performed either at The Rockefeller University (New York, NY) or at Macrogen, Inc. (Seoul, Korea). In the interpretation of results, single locus variants (SLVs) and double locus variants (DLVs) of a specific clone were assumed to be genetically related (4, 36).

eBURST. Correlation between the sequence types (ST) of novel *S. pneumoniae* clones with all ST existent in the database was performed, using the eBURST software available at the MLST website (www.mlst.net).

RESULTS

The 1,285 DRPn strains were characterized by PFGE typing, antimicrobial susceptibility testing, and serotyping. Eighty clones were identified by PFGE typing, of which 26 clones had five or more isolates and accounted for 1,195 (93%) DRPn isolates (Fig. 1). Sixty-one strains representative of the 26 DRPn clones were selected for MLST according to the criteria described in Materials and Methods.

MLST. Data obtained by MLST and comparison of the results with the MLST database led us to classify the 26 clonal types into three groups: PMEN clones, unique clones, and novel international clones. PMEN clones are clones whose representatives have identical allelic profiles or are SLVs or DLVs of resistant international clones included in the PMEN; novel international clones are clones recovered in other countries (besides Portugal) and that are currently not included in the PMEN; unique clones are clones that appear to have been detected for the first time in this study.

PMEN clones. This group comprised 13 clonal types (based on PFGE classification) corresponding to 945 (74%) DRPn isolates. MLST results confirmed that 10 PFGE clonal types matched the following 10 PMEN clones: Spain ^{23F}-1, Spain ^{9V}-3, England ^{14}-9, Colombia ^{23F}-26, Sweden ^{15A}-25, Poland ^{6B}-20, Portugal ^{19F}-21, Greece ^{6B}-22, Spain ^{6B}-2, and Spain ^{14}-5 (Table 1). One additional clone PFGE pattern, QQ, was highly related (DLV) to Colombia ^{23F}-26 (FF). The remaining two PFGE patterns, HHH and AL, were different DLVs of Hungary ^{19A}-6, although expressing a different serotype (Table 1).

Different SmaI PFGE patterns with the same or highly related ST are probably the result of rapid microevolution attributed to *S. pneumoniae* (12), resulting in alteration of the band pattern (changing band weights by changing restriction sites) though not changing the sequence of the housekeeping genes included in the MLST scheme.

Capsular switch evidence was observed for strains belonging to the following six PMEN clones: Spain ^{23F}-1, expressing serotype 19A; Colombia ^{23F}-26, expressing serotypes 15B and 6A; Sweden ^{15A}-25, expressing serotypes 19F and 19A; Hungary ^{19A}-6, expressing serotype 6B; Greece ^{6B}-22, expressing serotype 6A; and Spain ^{9V}-3, expressing serotypes 14 and 9A. A nontypeable isolate of Spain ^{9V}-3 was also found.

Interestingly, a single isolate of serotype 6A with PFGE pattern H—characteristic of clone Portugal ^{19F}-21—had ST 1152, which shares only three of seven alleles with the typical ST of this clone. This result was confirmed and, in our study, it is the single case where PFGE was a less discriminatory typing technique than MLST.

Unique clones. Four clones (PFGE patterns AR, AU, NB, and NC)—two of serotype 19A and two nontypeable—were assigned new ST (890, 1151, 1153, 1156, and 1278) (Table 2). No isolates with the same ST, neither SLVs nor DLVs, were available in the MLST database, with the exception of one strain with PFGE pattern AR (ST 888), which was formerly determined for one isolate from our previous studies (strain PT1804b).

Novel international clones. Nine clonal types were found to be distributed internationally through comparison of our results with those available in the MLST database. None was related to the 26 PMEN clones (approved up to December 2004 [http://www.sph.emory.edu/PMEN/]). Seven of these clonal types (AG, AQ, MM, N, NNN, T, and VVV) had been found in previous studies by our group (1996 to 1999) (27, 36–38), while the remaining two (AO and AY) were found for the first time in Portuguese day-care centers during this study (Table 3).

Clonal type AG (serotype 33F): ST 717. This clonal type was first detected in 1999 in the Lisbon area (27). This clone represents 2.0% of the total DRPn detected between 2001 and 2003. According to the MLST database, strains of the same ST were detected in Scotland in 2003 and found to be causative of invasive disease. A nontypeable DLV isolate, also of invasive

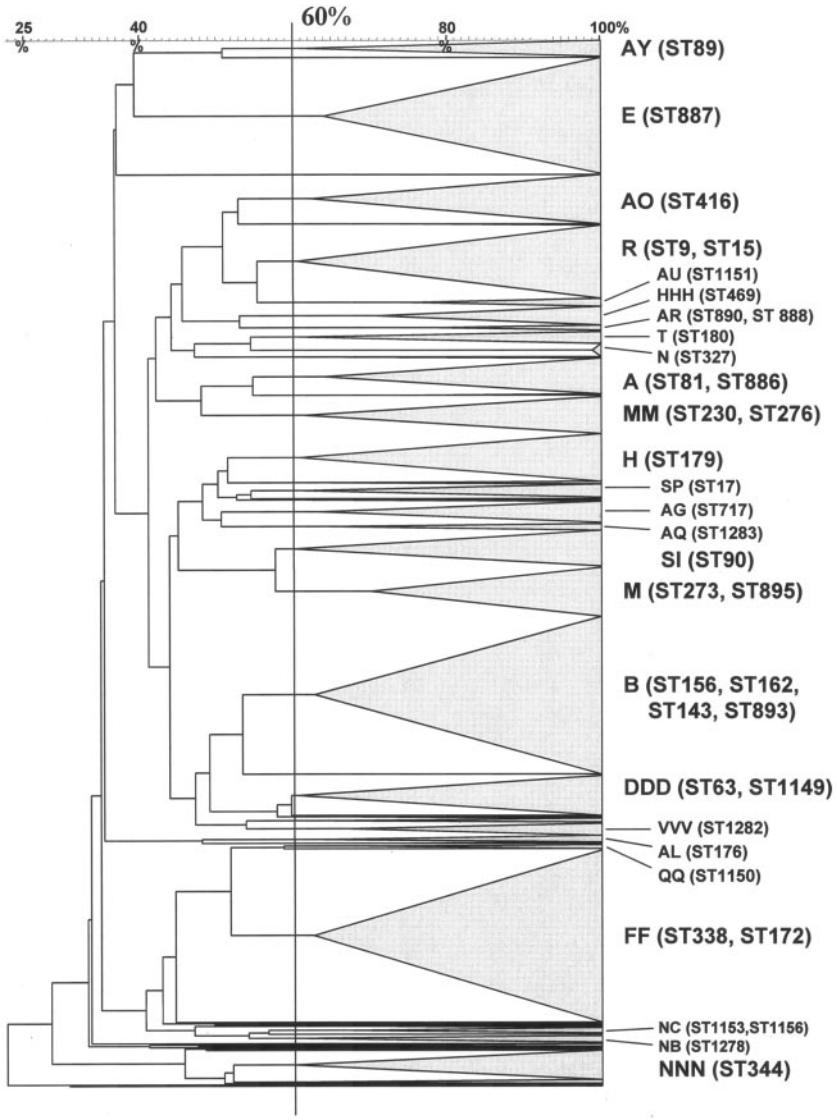

FIG. 1. Unweighted pair group method with arithmetic mean dendrogram of the 1,285 DRPn PFGE patterns using the Jaccard coefficient. Bionumerics comparison settings were 0% optimization and 1.4% tolerance. Each cluster tree was collapsed. The 60% similarity cutoff value is shown. Capital letters refer to PFGE nomenclature of the major clonal types. In parentheses is the MLST determined for selected isolates within a PFGE clone group.

origin, was found in the United States. SLVs were recovered during our studies carried out in 1999 (27).

Clonal type AO (serotype 19A): ST 416. Clonal type AO had not been detected in previous studies. This was the most frequent clonal type within the group of novel international clones, accounting for 4.1% of all DRPn. Identical allelic profiles and serotypes were detected in strains of carriage and invasive origin from the United Kingdom in 1999 to 2000. An SLV with the same capsular type was isolated in Greece. Several DLVs were found in the MLST database. Their origins were The Netherlands, Iceland (39), England, Scotland, Hungary, and the United States, and isolation dates ranged from 1987 through 2003. The majority of the isolates were from serotype 19A, but serotype 15B and serogroups 6 and 19 were also identified.

Clonal type AQ (serotype 19F): ST 1283. This clonal type was detected in 1999 in Lisbon day-care centers (27). It was attributed a new ST and was found to be a DLV of isolates having the same serotype, recovered in Malaysia and Brazil in 2000, both of which were associated with invasive disease.

Clonal type AY (serotype 19F): ST 89. Clonal type AY was not identified by our group in previous studies. The ST of this clonal type was detected in 1997 in Spain. It is genetically related to isolates recovered in Denmark, Italy (8), Spain, and Portugal (27). It is an SLV of a minor multiresistant Spain 19F clone (ST 88). Isolation dates of these foreign isolates ranged from 1987 to 1999, with the majority of the isolates being from invasive origin. All isolates were of serotype 19F.

Clonal type MM (serotype 19A): ST 230 and 276. This clonal type has been isolated from Portuguese day-care centers at-

TABLE 1. Properties of representatives of Pneumococcal Molecular Epidemiology Network clones detected in this study

Strain code	PG MIC (μg/ml)	Antibiotic resistance[a]	Serotype	PFGE pattern[b]	ST	Description
542	1.5	C, TE, SXT, TX	23F	A	886	SLV of Spain ^{23F}-1
4450	0.75	C, TE, SXT	23F	A	81	Spain ^{23F}-1
2574	0.5	C, TE, SXT	19A	A	81	Spain ^{23F}-1
1570	0.75	SXT	14	B	156	Spain ^{9V}-3
2807	2	E, DA, TE, SXT, TX	14	B	893	DLV of Spain ^{9V}-3
2737	1.5	E, DA	14	B	143	DLV of Spain ^{9V}-3
4140	0.047	E, DA, TE, SXT	9V	B	162	SLV of Spain ^{9V}-3
1683	1.5	SXT	NT[c]	B	156	Spain ^{9V}-3
952	0.023	E	14	R	9	England ^{14}-9
3451	0.5	E, DA, SXT	14	R	15	SLV of England ^{14}-9
1309	0.094		23F	FF	338	Colombia ^{23F}-26
2844	0.094		23F	FF	338	Colombia ^{23F}-26
2901	0.094		15B	FF	172	SLV of Colombia ^{23F}-26
1304	0.094		6A	QQ	1150	DLV of Colombia ^{23F}-26
3831	0.094		6A	QQ	1150	DLV of Colombia ^{23F}-26
1730	0.19	E, DA, TE	15A	DDD	63	Sweden ^{15A}-25
464	0.125	E, DA, TE	19F	DDD	1149	SLV of Sweden ^{15A}-25
1331	0.023	E, DA, TE	19A	DDD	63	Sweden ^{15A}-25
3919	0.094	E, DA, TE	6B	E	887	SLV of Poland ^{6B}-20
1605	0.094	E, DA, TE	6B	E	887	SLV of Poland ^{6B}-20
1282	0.016	E, DA, TE	19F	H	179	SLV of Portugal ^{19F}-21
4099	0.047	E, DA, TE	19F	H	179	SLV of Portugal ^{19F}-21
3026	0.012	C, E, DA, TE, SXT	6B	M	895	SLV of Greece ^{6B}-22
3536	0.016	C, E, DA, TE, SXT	6B	M	273	Greece ^{6B}-22
1175	0.023	C, E, DA, TE, SXT	6A	M	273	Greece ^{6B}-22
3104	1	C, E, DA, TE, SXT	6B	SI	90	Spain ^{6B}-2
2236	0.75	C, E, DA, TE, SXT	6B	SI	90	Spain ^{6B}-2
4034	1	C, E, DA, TE, SXT	14	SP	17	SLV of Spain ^{14}-5
2667	1.5	E, DA, TE, SXT	14	SP	17	SLV of Spain ^{14}-5
2703	0.023	E	6B	HHH	469	DLV of Hungary ^{19A}-6
2743	0.016	E	6B	HHH	469	
3572	0.023	E, DA, TE	6B	AL	176	DLV of Hungary ^{19A}-6
4058	0.023	E, DA, TE	6B	AL	176	DLV of Hungary ^{19A}-6

[a] Abbreviations: C, chloramphenicol; E, erythromycin; DA, clindamycin; TE, tetracycline; SXT, sulfamethoxazole-trimethoprim; TX, ceftriaxone; PG, penicillin. SLV, single-locus variants; DLV, double-locus variants.

[b] Numbers of isolates with each PFGE clonal type were as follows: A, 42; B, 184; R, 78; FF, 200; QQ, 5; DDD, 46; E, 135; H, 54; M, 119; SI, 44; SP, 10; HHH, 22; and AL, 6.

[c] NT, nontypeable.

tendees since 1997 (38). This clone represented 3.4% of all DRPn, being the second most frequent clonal type among the novel international clones. Evidence of diverse capsular switch was observed, since strains of serotypes 19F, 20, and 24F were detected. Isolates with the same ST were recovered in Denmark, Italy (29), and Sweden (40). These also had different serotypes: 14, 24F, and 19F, respectively. When searching the MLST database for SLVs and DLVs of this ST, a variety of isolates from The Netherlands, Poland, Norway, Sri Lanka, Sweden, India, the United States, Kenya, Finland, Australia, and Turkey was detected. Serotype diversity was also present in these latter isolates, where capsular types 23F, 19A, 19F, 14, and 3 were detected. The majority of these isolates were of invasive origin.

Clonal type NNN (nontypeable): ST 344. Clonal type NNN was first detected in 1997 as previously reported (38). Isolates having the same ST were also recovered in Norway, Australia, and Poland, from invasive origin and carriage. An SLV was recovered in the United States from a noninvasive disease, and DLVs were found in Finland, England, and Portugal in our previous studies (27). These isolates were also nontypeable and were all from carriage.

Clonal type N (serotype 6A): ST 327. This clonal type was first detected in 1996 in day-care centers of Lisbon (7). Isolates with identical MLST allelic profiles were recovered in England and Brazil from carriage and invasive sources, respectively. Invasive SLVs were recovered during 1982 in The Netherlands and during 1998 in the United States. Carriage SLV and DLV

TABLE 2. Properties of representatives of unique and novel international clone groups

Strain code	PG MIC (μg/ml)	Antibiotic resistance[a]	Serotype	PFGE pattern[b]	Allele							ST
					aroE	*gdh*	*gki*	*rec*	*spi*	*xpt*	*ddl*	
Unique clones												
2808	0.023	TE, SXT	19A	AR	8	74	19	15	6	40	26	888
2143	0.023	TE, SXT	19A	AR	8	74	4	15	6	40	27	890
3171	0.094		19A	AU	7	60	9	8	6	3	29	1151
1620	0.094		19A	AU	7	60	9	8	6	3	29	1151
1817	0.125	SXT	NT[c]	NB	2	6	4	29	91	19	147	1278
4812	0.19	SXT	NT	NB	2	6	4	29	91	19	147	1278
3201	4	E, DA, TE, SXT, TX	NT	NC	2	13	2	29	91	19	141	1153
5002	0.047	E, DA, TE	NT	NC	2	13	2	29	91	19	59	1156
Novel international clones												
2655	0.012	E, DA	33F	AG	5	35	29	1	45	39	18	717
2673	0.016	E, DA	33F	AG	5	35	29	1	45	39	18	717
2757	0.016	E, TE	19A	AO	1	13	14	4	17	51	14	416
2752	0.016	E, DA, TE	19A	AO	1	13	14	4	17	51	14	416
1809	0.023	E, DA, TE	19F	AQ	15	16	96	5	6	1	26	1283
1750	0.023	E, DA, TE	19F	AQ	15	16	96	5	6	1	26	1283
4313	0.38	C, E, DA, TE, SXT	19F	AY	5	5	7	7	8	5	1	89
3815	0.38	C, E, DA, TE, SXT	19F	AY	5	5	7	7	8	5	1	89
4914	0.023	E, DA, TE	3	T	7	15	2	10	6	1	22	180
4076	0.023	E, DA, TE	3	T	7	15	2	10	6	1	22	180
2436	0.5	E, DA, TE	19A	MM	2	19	2	17	6	22	14	276
2679	0.38	E, DA, TE	19A	MM	12	19	2	17	6	22	14	230
4815	0.125	E, DA, TE	19F	MM	12	19	2	17	6	22	14	230
2942	0.75	E, DA, TE	24F	MM	12	19	2	17	6	22	14	230
5137	0.38	E, DA, TE	20	MM	12	19	2	17	6	22	14	230
320	0.023	E, SXT	6A	N	1	5	7	12	10	1	14	327
3651	0.023	E, SXT	6A	N	1	5	7	12	10	1	14	327
780	0.125	E, DA, TE, SXT	NT	NNN	8	37	9	29	2	12	53	344
944	0.125	E, TE, SXT	NT	NNN	8	37	9	29	2	12	53	344
4737	0.016	E, DA	10A	VVV	7	7	4	2	10	1	27	1282
3700	0.016	E, DA	10A	VVV	7	7	4	2	10	1	27	1282

[a] Abbreviations: C, chloramphenicol; E, erythromycin; DA, clindamycin; TE, tetracycline; SXT, sulfamethoxazole-trimethoprim; TX, ceftriaxone; PG, penicillin.
[b] Numbers of isolates with each PFGE clonal type were as follows: AR, 7; AU, 9; NB, 10; NC, 7; AG, 26; AO, 53; AQ, 8; AY, 20; T, 16; MM, 44; N, 15; NNN, 21; and VVV, 14.
[c] NT, nontypeable.

strains were found in England, and one invasive DLV was recovered in Scotland. The isolates were of serotype 6A or had only the indication of serogroup 6.

Clonal type VVV (serotype 10A): ST 1282. This PFGE pattern was first detected during 1998 in Lisbon day-care centers where the majority of the strains found were drug susceptible (7). A new ST was attributed to this clone. Invasive SLV isolates from Spain, Scotland, and Portugal were found and had the same capsular type, 10. DLV of this ST were recovered in the United Kingdom, Finland, USA and Portugal, being the majority from invasive origin. The serotypes of these isolates were 10A and 6A.

TABLE 3. Number of isolates of novel international clones recovered from 1996 to 2003

PFGE pattern	1996	1997	1998	1999	2001	2002	2003
AG				5	7	16	3
AO					27	16	10
AQ				1	7		1
AY					5	9	6
T			4	1	1	10	5
MM		2	3		7	25	12
N	4	3			13	2	
NNN		1	3	6	15	3	3
VVV			21[a]			2	12

[a] Nineteen strains were drug susceptible.

TABLE 4. Antimicrobial resistance patterns of novel international clones and dissemination in day-care centers[a]

PFGE pattern	Serotype(s)	No. of isolates	Antibiotype		No. of DCC where clone was present (out of 13)
			MIC of penicillin (μg/ml)	Other resistance markers	
MM	19A (29), 19F (3), 20 (3), 24F (9)	44	0.125–0.75	C, E (44), DA (44), TE (44), SXT (14)	6
N	6A	15	Susceptible	E (15), SXT (15)	1
NNN	NT	21	0.094–1	E (21), DA (17), TE (21), SXT (21), TX	7
VVV	10A	14	Susceptible	E (14), DA (14), TE (4)	1
AG	33F	26	Susceptible	E (26), DA (26), TE (2)	8
AO	19A	53	Susceptible	E (53), DA (53), TE (53)	2
AQ	19F	1	0.25	SXT	1
		7	Susceptible	E (7), DA (7), TE (7)	1
AY	19F	19	0.094–0.5	C (19), E (18), DA (18), TE (18), SXT (18), TX	5
T	3	1	0.38	E, DA, TE, SXT	1
		15	Susceptible	E (15), DA (15), TE (15)	3

[a] Abbreviations: C, chloramphenicol; E, erythromycin; DA, clindamycin; TE, tetracycline; SXT, sulfamethoxazole-trimethoprim; TX, ceftriaxone; NT, nontypeable; DCC, day-care centers. Numbers between parentheses indicate numbers of isolates.

Clonal type T (serotype 3): ST 180. This clonal type was identified in 1998 in Portuguese day-care centers (38). Isolates with the same ST were found in The Netherlands, the United Kingdom, Denmark, Spain (31), Canada, and Taiwan. SLVs of this clone were found in the United Kingdom, Denmark, Taiwan, the United States, and Portugal. A unique DLV was recovered in Finland. This clone was first recovered in 1984 in The Netherlands, and the vast majority of the foreign isolates were of invasive origin. All isolates were of serotype 3. This clone is presently being proposed to PMEN for acceptance by P. Hermans and B. Spratt (L. McGee, Minutes of the 8th Meeting of the PMEN, Helsinki, Finland, 12 May 2004).

Antibiotype of novel international clones. The novel international clones comprised 216 DRPn strains (16.8% of the DRPn), the majority being disseminated in several day-care centers (Table 4). Nearly all strains were resistant to erythromycin (99.1%), which was often associated with resistance to clindamycin (90.3%), tetracycline (76.4%), and occasionally sulfamethoxazole-trimethoprim (32.4%) and chloramphenicol (9.3%). Only three clones (MM, AY, and NNN) out of nine had intermediate resistance to penicillin, representing 40% of the novel international isolates. The remaining clones were susceptible to penicillin, with the exception of single isolates of clones AQ and T (Table 4).

eBURST. To assess the distribution of the ST of the novel clones within the entire database of MLST, we performed an eBURST analysis (11). In eBURST, the correlation of ST is based on the allelic profiles, where the ancestor within a group is the ST with more SLVs. eBURST analysis was performed for our clones together with the entire *S. pneumoniae* ST database, and isolates with related ST (5/7 shared alleles) were grouped. Eighty-six groups were obtained in the MLST database, where group 1 was the major one with 1,037 isolates; the following eight groups ranged between 114 and 26 isolates. We observed that seven of the nine novel international clones were dispersed into seven groups. This was in sharp contrast with the 11 PMEN clones identified in our study: they were all clustered in group 1. Two of the novel international clones (ST 180 and ST 416) were also in group 1. Interestingly, the ST of two of the four unique clones were singletons. One of the remaining unique clones belonged to group 2 (the same as clone AQ, ST 1283), and the other one (clone NC) and its two ST (ST 1153 and 1156) formed a new group.

DISCUSSION

Children constitute the main reservoir of pneumococci and are probably the vector for spread of the bacterium to adults (36, 37). Characterization of the nasopharyngeal pneumococci in children is important for monitoring trends in antimicrobial resistance and for a better understanding of the population biology of this pathogen.

PMEN clones were the most frequently isolated group in our study, similarly to what has been reported in previous studies in the same settings (36, 37). This group accounted for 79.1% of the 1,195 DRPn considered in this study (Table 1). By PFGE, serotyping, and antimicrobial susceptibility testing it was possible to assign 76.3% of the isolates to representatives of PMEN clones. MLST not only confirmed these results but also identified three additional PFGE clones as having an ST highly related to those of these international clones. The assignment of different PFGE patterns to the same MLST highlights the genetic evolution of these clones: their genotypes are changing, leading to an increasing number of PFGE subtypes and, eventually over time, to the emergence and assignment of a distinct PFGE pattern. This phenomenon reflects the high genomic plasticity of this pathogen (5). For instance, acquisition or loss of resistance genes (for example, macrolide and tetracycline resistance determinants) associated with integrative mobile elements, such as transposons, may alter the original PFGE pattern of an isolate by originating new band profiles.

By comparing the allelic profiles of our isolates with the ones included in the MLST database, we have found that several of the clones we characterized in the Lisbon area were internationally disseminated and thus were named as novel international clones. Besides this information, data on the clinical sources and capsular types of those identical/highly related isolates (SLVs and DLVs) were also retrieved.

A common property of these novel international drug-resistant pneumococcal clones was resistance to erythromycin often associated with resistance to clindamycin and tetracycline (Ta-

TABLE 5. Clones to be proposed for Pneumococcal Molecular Epidemiology Network inclusion

ST	Serotype	Antimicrobial resistances[a]	PFGE pattern
327	6A	E, SXT	N
230	19A	PGI, E, DA, TE	MM
416	19A	E, DA, TE	AO
1282	10A	E, DA	VVV
717	33F	E, DA	AG
344	NT[b]	PGI, E, DA, TE, SXT	NNN
1283	19F	E, DA, TE	AQ
89	19F	PGI, C, E, DA, TE, SXT	AY

[a] Abbreviations: PGI, penicillin intermediate resistance (0.1 μg/ml ≤ MIC < 1.5 μg/ml); C, chloramphenicol; E, erythromycin; DA, clindamycin; TE, tetracycline; SXT, sulfamethoxazole-trimethoprim.
[b] NT, nontypeable.

ble 4). Interestingly, only three of these clones had intermediate resistance to penicillin, corroborating previous findings that erythromycin-resistant and penicillin-susceptible clones are emerging worldwide (37, 41). This is probably related to the increase of macrolide-resistant strains observed in recent years (18, 19, 32).

The introduction of the 7-valent pneumococcal conjugate vaccine may also contribute to the emergence of new or less frequent drug-resistant pneumococci expressing capsular serotypes not covered by the vaccine (14, 33), since this vaccine was conceived to target seven of the most common serotypes responsible for invasive disease (4, 6B, 9V, 14, 18C, 19F, and 23F), particularly in the United States. Recent studies reported its high efficacy in reducing invasive disease (2, 47) and there is evidence that it also reduces the nasopharyngeal carriage of vaccine-type strains (6, 13, 14, 17). However, an increasing frequency in carriage of nonvaccine-type strains has been observed by some and this may lead to the emergence of nonvaccine-type drug-resistant clones (14, 28, 46). The behavior and the disease potential of these nonvaccine types is still unknown, but it is possible that diseases caused by nonvaccine serotypes will increase over time (30). In fact, in one study the number of otitis media cases caused by nonvaccine types increased after introduction of the vaccine (34).

In this study the majority of the novel international clones have nonvaccine serotypes unusual in carriage: serotypes 3 (clonal type T), 33F (clonal type AG), 10A (clonal type VVV), 20 and 24F (clonal type MM), and nontypeable (NNN), all of which are identical or highly related to invasive isolates according to the data provided by the MLST database. Only two of the nine clones (AY and AQ) had a vaccine serotype, 19F (Table 2). Serotypes 19A and 6A were also present in this group, and although these serotypes may be considered to be covered by the vaccine due to cross-reactivity with serotypes 19F and 6B, respectively (45, 49), a recent study did not find conclusive evidence that serotype 19A was adequately covered by the 7-valent conjugate vaccine (47).

eBURST performed on novel international clone ST illustrate the diversity of these clones as the nine novel international clones were dispersed through eight distinct groups, suggesting that these clones have distinct evolutionary origins.

Our results reinforce the idea that careful monitoring is becoming increasingly important, especially regarding the emergence of nonvaccine-type DRPn strains with unusual genetic backgrounds.

We propose that the novel international clones should be considered for inclusion in the PMEN (Table 5). The increasing frequency of these clones (27, 36–38) with disease potential might be an indication of the invasive multidrug-resistant international clones of the near future.

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