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Genetic Diversity and Clonal Patterns among Antibiotic-Susceptible and -Resistant *Streptococcus pneumoniae* Colonizing Children: Day Care Centers as Autonomous Epidemiological Units

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Characterization by antibiotype of the 1,096 *Streptococcus pneumoniae* recovered from 2,111 nasopharyngeal samples of children attending 16 day care centers (DCCs) in Lisbon, Portugal, and molecular typing of 413 drug-resistant pneumococci (DRPn) and 89 fully drug-susceptible pneumococci (DSPn) has allowed several conclusions. (i) There was an increase in the frequency of DRPn colonizing children in DCCs from 40% in 1996 to 45% in 1997 to 50% in 1998. (ii) Drug resistance spread by cross-transmission of DRPn clones. A few (8 out of 57) DRPn clones were repeatedly isolated from a large number of children in several DCCs and during each period of surveillance, suggesting the epidemic nature of these clones, which included lineages representing internationally spread *S. pneumoniae* clones. (iii) Dissemination of resistance determinants among pneumococci colonizing the nasopharynx occurred. Association of identical pulsed-field gel electrophoresis patterns with diverse antibiotypes among pneumococci colonizing children suggests that the high prevalence of DRPn involves not only cross-transmission of resistant strains but also dispersal of resistance genes through recombinational mechanisms. (iv) DCCs are autonomous epidemiological units. Among the 413 DRPn, 57 different lineages were detected; these lineages were dispersed among the 16 DCCs to produce unique microbiological profiles for each of the DCCs. Higher genetic diversity and less sharing of clonal types were observed among the DSPn.

Streptococcus pneumoniae has remained a major cause of potentially life-threatening infections, for which children of preschool age are at particularly high risk. Due to a variety of social and cultural factors, an increasing proportion of children of preschool age are now attending day care centers (DCCs) in several countries. Through this social trend, DCCs have emerged as novel epidemiological entities comprising a human population with unique behavioral traits, incompletely matured immune systems, and susceptibility to both viral and bacterial upper-respiratory infections. Because of the high incidence of respiratory disease and the large number of prescriptions of antimicrobial agents, bacteria colonizing children of DCC age are under extreme antibiotic pressure, and this may be one of the reasons why attendance in DCCs is a risk factor for the carriage of drug-resistant *S. pneumoniae* (for a review see reference 15). Although much work on the isolation of *S. pneumoniae* from healthy children in DCCs has been done, few studies have systematically applied molecular typing techniques (2–4, 23, 29) in the same group of DCCs for surveillance periods over several years to identify trends in the composition and resistance level of the pneumococcal flora colonizing the nasopharynx. Such methods and study design are essential for the understanding of the epidemiological role of DCCs in the spread of drug-resistant strains and are also needed in the choice of intervention strategies to decrease carriage of antimicrobial-resistant strains.

With these motives in mind, in 1996, we began a 2-month-per-year surveillance study of the nasopharyngeal flora of children attending a group of selected DCCs in Lisbon, Portugal, with special attention paid to drug-resistant isolates. By the end of the third surveillance period in 1998, 16 DCCs and 1,617 children had participated in the study, generating 2,111 nasopharyngeal samples that were screened for *S. pneumoniae* and other respiratory pathogens. *S. pneumoniae* was detected in more than half (1,096) of the 2,111 nasopharyngeal samples. Results of the characterization of resistant pneumococci and *Haemophilus influenzae* and *Moraxella catarrhalis* recovered during the first year of the surveillance study in 1996 have already been described (8).

The aim of the study described in this paper was to extend the 1996 observations to the large collection of drug-resistant pneumococcal isolates (DRPn) recovered in the second and third periods of surveillance and to identify temporal changes in the serotype, antibiotype, and molecular type of the flora that may have occurred. In order to obtain a more complete picture of the epidemiology of the pneumococcal flora, 89 *S. pneumoniae* isolates fully susceptible to all antimicrobial agents and recovered from the nasopharynges of children attending the three largest DCCs in 1996 were also characterized by molecular typing techniques.

MATERIALS AND METHODS

DCCs and surveillance periods. According to the last official census in 1991, 7,500 children with ages ranging from 6 months to 6 years attended DCCs in

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Lisbon, the capital of Portugal. Our main goal was to obtain a representative sample of the children attending DCCs in Lisbon. Thus, DCCs were selected in order to represent different geographic areas and a spectrum of different demographic and social backgrounds of the city of Lisbon. Although the set of DCCs selected varied from year to year, these criteria were achieved in each surveillance period. In 1996, a group of 586 children attending seven DCCs (DCCs 1 to 7) participated in the study (8). Corresponding figures were 745 children in 12 DCCs (DCCs 1, 2, 4, and 6 to 14) in 1997 and 780 children in 12 DCCs (DCCs 1, 2, 4 to 9, 11, and 14 to 16) in 1998. Thus, 8 to 10% of the target population participated in the study. Nasopharyngeal samples were recovered in the winter months of February and March of 1996, 1997, and 1998. In Portugal, as in many other countries, respiratory infections have a higher incidence in winter and so does antibiotic prescription. These late-winter months were selected in order to provide information about the effect of infectious diseases and antimicrobial pressure on nasopharyngeal carriage.

At each sampling period one sample was obtained from each participating child. A total of 1,211 children were enrolled at only one of the sampling periods, 298 children were enrolled twice, and 98 children participated in the three sampling periods. The total number of nasopharyngeal samples obtained was 2,111.

Approval for the study was obtained from the Ministry of Education and the directors of the DCCs. Signed informed consent was obtained from the parents of all participating children.

Isolation of *S. pneumoniae* from nasopharyngeal samples. Routinely, only one colony of *S. pneumoniae* was purified from each nasopharyngeal sample, and the culture obtained was frozen and used in further studies. *S. pneumoniae* isolates were identified by standard procedures as previously described (8). Isolates were frozen in glass vials containing Mueller-Hinton broth (Difco, Detroit, Mich.) and 15% (vol/vol) glycerol (Merck, Darmstadt, Germany) and kept at −70°C.

Growth conditions. *S. pneumoniae* was grown in a synthetic medium (17) supplemented with yeast extract (0.1% [wt/vol] final concentration; Difco) without aeration at 37°C or on tryptic soy agar (Difco) supplemented with 5% sheep blood and incubated in a 5% CO_2 atmosphere at 37°C.

Antimicrobial susceptibility testing. Testing of susceptibility to chloramphenicol, erythromycin, clindamycin, tetracycline, and sulfamethoxazole-trimethoprim (SXT) was performed using the Kirby-Bauer technique, according to the National Committee for Clinical Laboratory Standards recommendations and definitions (22). MICs of penicillin and ceftriaxone were determined with the E-test (AB Biodisk, Solna, Sweden). In the interpretation of decreased penicillin susceptibility of *S. pneumoniae*, isolates were arbitrarily divided into "low-level" (MIC between 0.1 and 0.5 μg/ml) and "high-level" (MIC ≥ 0.5 μg/ml) resistance strains. While this grouping differs from the current clinical microbiological breakpoints, it fits better the molecular mechanism of penicillin resistance (7).

Serotyping. Capsular types and groups were determined by the Quellung reaction at The Rockefeller University or by coagglutination at the National University Hospital, Reykjavik, Iceland, using Danish typing sera (16, 19).

Molecular typing. Pneumococcal strains resistant to at least one of the antimicrobial agents tested were selected for molecular typing, with the exception of strains that were resistant only to SXT (27 in 1997 and 26 in 1998), which were not included in the molecular typing. Previous studies demonstrated a high degree of diversity among SXT-resistant strains similar to that seen among fully drug-susceptible pneumococcal isolates both in terms of serotypes and molecular types, suggesting frequent independent emergence of resistance to this drug (8).

In order to compare the diversity among DRPn to that among drug-susceptible pneumococci (DSPn), a group of *S. pneumoniae* isolates (89 strains) fully susceptible to all antimicrobial agents tested was also included in the molecular characterization. These strains represented all the DSPn recovered from the three largest DCCs (DCCs 1, 2, and 7) studied in 1996 (95 isolates) with the exception of six isolates which could not be revived. The three DCCs were selected due to their high number of attendees, which generated several isolates (149 in DCC 1, 98 in DCC 2, and 148 in DCC 7) enabling, upon analysis of the typing results, a better understanding of the pneumococci circulating in these settings.

Preparation of chromosomal DNA. This method was adapted from an earlier procedure (25). A 6-ml culture of early-stationary-phase cells was harvested, washed in 1 ml of phosphate buffer (50 mM KPO_4, pH 7), and resuspended in 200 μl of the same buffer. Concentrations were adjusted to an optical density of 5.0 at 620 nm. This cell suspension was then diluted 1:1 with 150 μl of 1.5% low-gelling-temperature agarose (SeaPlaque; FMC Bioproducts, Rockland, Maine) in phosphate buffer; disks of 20 μl were allowed to solidify for 5 min at −20°C. The cells were lysed by incubation of the disks at 37°C for 3 h with 50 μg of RNase I in 1 ml of EC buffer (6 mM Tris [pH 8], 1 M NaCl, 0.1 M EDTA [pH 8], 0.2% deoxycholate, 0.5% Sarkosyl, 0.5% Brij 58). The disks were next incubated in a solution of proteinase K (1 mg/ml) in ES buffer (0.5 M EDTA [pH 9], 1% Sarkosyl) at 50°C for 17 h. The agarose disks were washed five times in 13 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 7.5]) for 1 h with gentle agitation. The DNA was then considered purified and was stored in ca. 1 ml of TE buffer at 4°C.

DNA restriction and PFGE. DNA was digested with 20 U of *Sma*I (New England Biolabs, Beverly, Mass.), and pulsed-field gel electrophoresis (PFGE) was performed in a CHEF DR-II (contour-clamped homogeneous electric field) apparatus (Bio-Rad, Birmingham, United Kingdom) for 23 h. The running parameters were as follows: initial pulse, 5 s; final pulse, 35 s; voltage, 200 V; temperature, 11.3°C. Standard methodologies were used for staining and photographing the gels (24). Strain R6, a derivative of nonencapsulated strain R36A (1), and PFGE lambda marker (New England Biolabs) were used as molecular weight standards. Analysis of *Sma*I macrorestriction profiles was done by visual inspection of the patterns using currently accepted criteria (27). PFGE patterns generated by DRPn were assigned uppercase letters and those generated by DSPn were assigned lowercase letters. The particular PFGE patterns, which were already detected in 1996, received the same letter assignments in the 1997 and 1998 samples.

Control strains. *S. pneumoniae* strain ATCC 49619 was used as control strain in the antimicrobial susceptibility tests. *S. pneumoniae* strain M13P01, a representative of the penicillin-resistant French/Spanish clone (of capsular type 9V or 14) (12) and strain Clev2, a representative of the multidrug-resistant serotype 23F Spanish/U.S. clone (21), were obtained from the strain collection of the Laboratory of Microbiology, The Rockefeller University, and were included in the molecular typing analysis.

RESULTS

Increasing frequency of DRPn. During the 2-month surveillance periods in 1996 (8), 1997, and 1998, the overall carriage rates of *S. pneumoniae* were 47 (277 isolates), 48 (354 isolates), and 60% (465 isolates), respectively. The frequency of DRPn colonizing children has gradually increased from 40% (111 of 277 isolates) in 1996 to about 45% (161 of 354 isolates) in 1997 to close to 50% (231 of 465 isolates) in 1998.

Of the 277 pneumococci isolated in 1996, 20% had decreased susceptibility to penicillin: 11% had high-level resistance (MIC ≥ 0.5 μg/ml; 31 isolates), and 9% had low-level resistance (0.1 ≤ MIC < 0.5 μg/ml). Rates of resistance to other antimicrobial agents were 8% for chloramphenicol, 22% for tetracycline, 33% for SXT, and 6% for ceftriaxone. Concerning macrolide-lincosamide resistance 17% of the isolates were resistant to erythromycin and 14% were resistant to clindamycin. The most frequent DRPn serogroups/serotypes were 6 (39%), 23 (22%), 19 (20%), and 14 (12%) (8).

Of the 354 pneumococci isolated in 1997, 30% had decreased susceptibility to penicillin: 8% had high-level resistance (27 isolates), and 22% had low-level resistance. Rates of resistance to other antimicrobial agents were 7% for chloramphenicol, 21% for tetracycline, 23% for SXT, and 3% for ceftriaxone. Concerning macrolide-lincosamide resistance 18% of the isolates were resistant to erythromycin and 18% were resistant to clindamycin. The majority of DRPn were of serotypes 23F (28%), 6B (24%), 19F (11%), 19A (10%), 6A (10%), and 15 (6%).

Of the 465 pneumococci isolated in 1998, 26% had decreased susceptibility to penicillin: 13% had high-level resistance (61 isolates) and 13% had low-level resistance. Rates of resistance to other antimicrobial agents were 10% for chloramphenicol, 26% for tetracycline, 26% for SXT, and 6% for ceftriaxone. Concerning macrolide-lincosamide resistance 26% of the isolates were resistant to erythromycin and 19% were resistant to clindamycin. The most frequent DRPn serogroups/serotypes were 6 (26%), 23F (20%), 14 (17%), 19 (14%), 9V (10%), and 15 (3%).

Molecular typing of DRPn. Of the 111 DRPn isolated in 1996 (8), 16 strains were only resistant to SXT and 4 were not fully typed, leaving 91 strains which were used for comparisons with the second and third surveillance periods. Of the 161 DRPn isolated in 1997, 27 were not characterized further since they were only resistant to SXT (see Materials and Methods) and 3 strains were lost, leaving 131 DRPn for molecular typing. Of the 231 DRPn isolated in 1998, 26 were only resistant to SXT and 14 could not be revived, leaving 191 strains for molecular typing. Tables 1 (adapted from reference 8) to 3 show in detail the microbiological profiles of each DCC in 1996, 1997, and 1998, respectively: DRPn isolates are grouped on the

TABLE 1. Diversity of microbiological profiles among DRPn isolated in DCCs in 1996[g]

Penicillin resistance[b]	PFGE type	Capsular group/type	Antibiotype (resistant to[c]:)	Total no. of strains[g]:	No. of strains in DCC with clonal type						
					1	2	3	4	5	6	7
High	B	9V	P, SXT	1							1
	B	14	P, SXT, TX (2)	9	7	1		1			
	A	23F	P, C, TC, SXT, TX (14)	20			4	1		3	12
	nd[d]	6B	P, E, TC, SXT	—[e]		1					
Low	G	6B	P	1							1
	E	6B	P, E, CC, TC, SXT (1)	5		3					2
	H	19	P, SXT	1							1
	D	19/19A	P, SXT	11							11
	K	23	P, E, CC, TC, SXT	1			1				
	Unique[f]	6B	P, E, CC, TC, SXT	1							1
	Unique	19	P, E, CC, TC, SXT	1							1
	Unique	19	P, SXT	1	1						
	Unique	nd	P, E, SXT	—	1						
	K	nd	P, E, CC, TC, SXT	—	1						
	nd	6B	P, E, TC, SXT	—			1				
Susceptible[h]	M	6	C (2), E (16), CC (16), TC, SXT (14)	18	12		1				5
	L	6	E, CC, TC, SXT	8	8						
	R	14	E, CC, SXT	1	1						
	H	19	E, CC	3	2					1	
	Unique	6	E	1	1						
	Unique	6	TC, SXT	1					1		
	Unique	11	E, CC	2	2						
	Unique	11	E, SXT	1					1		
	Unique	14	TC	1			1				
	Unique	18	TC	1				1			
	Unique	19	E, CC	1	1						
	Unique	19	E, TC	1						1	

[a] Data taken from reference 8.
[b] High, MIC ≥ 0.5; low, 0.1 ≤ MIC < 0.5; susceptible, MIC < 0.1 μg/ml.
[c] P, penicillin; C, chloramphenicol; E, erythromycin; CC, clindamycin; TC, tetracycline; TX, ceftriaxone. Isolates resistant to only SXT were not included. Numbers in parentheses indicate numbers of isolates resistant to the antimicrobial agent (no number, all isolates were resistant).
[d] nd, not determined.
[e] —, clonal type was not determined since information on either serotyping or PFGE profile was not determined.
[f] Unique, each isolate had a unique PFGE type that was not found in any other isolate although some had a common antibiotype.
[g] There were a total of 91 strains.
[h] Susceptible, susceptible to penicillin and resistant to other drugs.

basis of a combination of PFGE type, capsular type, and antibiotype, and their representation (number) in each DCC is tabulated. During the three surveillance periods, a total of 57 PFGE types were detected in the DCCs.

Dissemination of epidemic clones in DCCs. Of the 57 PFGE types, a set of 8 PFGE types stood out since isolates with one of these patterns had been repeatedly recovered in more than one DCC, were identified in more than one surveillance period, and were generally able to colonize many children. These PFGE types were types A (69 strains), B (38 strains), E (22 strains), H (25 strains), M (64 strains), R (28 strains), FF (23 strains), and DDD (15 strains) (Table 4). Two of these recurrent clones (PFGE types A and B) had high-level resistance to penicillin (penicillin MIC ≥ 0.5 μg/ml); the common feature of five (PFGE types E, H, M, R, and DDD) of the remaining six clones was resistance to erythromycin accompanied by either low-level resistance or no resistance at all to penicillin. Strains with PFGE type FF had low-level resistance to penicillin and were susceptible to other antimicrobial agents. In most DCCs a large proportion of DRPn isolates belonged to some of these eight clones (Table 4). Next we shall describe the properties of these eight epidemic clones in more detail.

Two DRPn clones with high-level resistance to penicillin. Strains of PFGE type A were all of serotype 23F, had high-level resistance to penicillin, and were resistant to chloramphenicol, tetracycline, and SXT. A few isolates were, in addition, resistant to erythromycin and clindamycin. These strains were found to be representatives of the widely spread multidrug-resistant Spanish/U.S. 23F clone (21). This clone was detected in four centers (DCCs 3, 4, 6, and 7) in 1996 (8), in four centers in 1997 (DCCs 1, 7, 8, and 11), and in six centers (DCCs 1, 2, 7, 8, 15, and 16) in 1998.

All isolates of PFGE type B expressed capsular type 9V or 14, had high-level resistance to penicillin, and were also resistant to SXT. Isolates of this clonal type belonged to the internationally disseminated French/Spanish clone, which typically expresses capsular type 9V or 14 (12). This clone had been detected in four centers (DCCs 1, 2, 4, and 7) in 1996 (8), in two centers (DCCs 8 and 13) in 1997, and in five centers (DCCs 1, 2, 7, 15, and 16) in 1998.

Together, the Spanish/U.S. 23F (PFGE type A) and the French/Spanish 9V or 14 (PFGE type B) clones represented 97% (30 of 31 strains) of the highly penicillin-resistant isolates recovered in 1996 (8), 85% (23 of 27 strains) of those recovered in 1997, and 89% (54 of 61 strains) of those recovered in 1998.

Five DRPn resistant to macrolide antibiotics. Figure 1 shows the PFGE profiles of each of these macrolide-resistant clones and their properties, and their dissemination in DCCs is shown in Tables 1 to 4 and is also briefly described below.

TABLE 2. Diversity of microbiological profiles among DRPn isolated in DCCs in 1997

Penicillin resistance[a]	PFGE type	Capsular group/type	Antibiotype (resistant to[b]:)	Total no. of strains[c]:	No. of strains in DCC with clonal type											
					1	2	4	6	7	8	9	10	11	12	13	14
High	B	9V	P, SXT, TX	1						1						
	B	14	P, SXT, TX	1											1	
	DDD	15	P, E, CC, TC, SXT	2			2									
	MM	19F	P, E, CC, TC, SXT	2						2						
	A	23F	P, C, E (6), CC (6), TC, SXT, TX (10)	21	7				5	4			5			
Low	G	6B	P	8				7						1		
	M	6B	P, C (1), E, CC, TC, SXT (1)	2		2										
	E	6A/6B	P, E (11), CC (11), TC (11)	12		12										
	QQ	6A/6B	P	10		10										
	DDD	15	P, E, CC, TC	6			4			2						
	D	19A	P, SXT	13					13							
	FF	23F	P	14	7				6		1					
	AAA	NT[e]	P, E, CC, TC, SXT	4	4											
	NNN	NT	P, E, CC, TC, SXT	1		1										
	Unique[f]	6B	P, E, CC, TC, SXT (1)	2		1	1									
	Unique	19F	P	1											1	
	Unique	23F	P	1							1					
	Unique	23B	P, E, TC, SXT	1		1										
	Unique	Pool I	P, E, CC, TC, SXT	1						1						
	Unique	Pool G	P	1			1									
	Unique	NT	P, E, CC, TC, SXT	3	1	1				1						
Susceptible	E	6B	E, CC, TC	1					1							
	M	6B	C (2), E, CC, TC, SXT (6)	8	1				1	2	2		1	1		
	H	19F	E, CC, TC (4)	11	7		1	3								
	Unique	6A	E, CC, TC, SXT	1						1						
	Unique	6B	C, E, CC, SXT	1										1		
	Unique	6B	E	1											1	
	Unique	19F	E, CC, TC	1						1						
	nd[d]	nd	TC, SXT (1)	—[g]					1	1			1			

[a] See Table 1 for definitions of high and low resistance and susceptible.
[b] Drug abbreviations and parenthetical numbers are as defined for Table 1.
[c] The total number of strains was 131.
[d] nd, not determined.
[e] NT, nontypeable.
[f] Unique is as defined for Table 1.
[g] —, see definition in Table 1 footnotes.

Clone E (serotype 6A or 6B) was detected in DCCs 2 and 7 in 1996 and in 1997 and only in DCC2 in 1998. Twenty-one of the 22 isolates (95%) belonging to this clone had low-level resistance to penicillin and resistance to erythromycin, clindamycin, and tetracycline.

Clone M (serotype 6B) was detected in three centers (DCCs 1, 3, and 7) in 1996, in seven centers (DCCs 1, 2, 7, 8, 9, 11, and 12) in 1997, and also in seven centers (DCCs 1, 6, 7, 8, 9, 11, and 16) in 1998. Sixty-one of the 64 isolates (95%) of this lineage were susceptible to penicillin and resistant to erythromycin, clindamycin, tetracycline, and SXT.

Clone R (serotype 14) was detected in a single isolate from DCC 1 in 1996 and in four centers (DCCs 2, 7, 8, and 15) in 1998. Most strains (93%) were only resistant to erythromycin.

Clone DDD (serotype 15) was not detected in 1996 and was detected in two centers (DCCs 4 and 8) in 1997 and in three centers in 1998 (DCCs 2, 6, and 7). All strains were resistant to erythromycin, clindamycin, and tetracycline, and the penicillin MICs for them ranged from 0.1 to 0.5 μg/ml.

Clone H (serotype 19F) was recovered in three centers in 1996 (DCCs 1, 6, and 7), three centers in 1997 (DCCs 1, 4, and 6), and four centers in 1998 (DCCs 1, 2, 4, and 9). Although different antibiotypes were found, most isolates were susceptible to penicillin and resistant to erythromycin and clindamycin.

A DRPn clone with low-level resistance to penicillin. Clone FF (serotype 23F) was not detected in 1996 and was detected in three centers in 1997 (DCCs 1, 7, and 9) and 1998 (DCCs 5, 6, and 7). Strains of this type had low-level resistance to penicillin and were susceptible to other antimicrobial agents. Their PFGE profile is shown in Fig. 1B.

DRPn clones unique to each DCC. Although the eight clones referred to above generally accounted for most of the DRPn isolates in a DCC, most centers (six DCCs in 1996, eight DCCs in 1997, and seven DCCs in 1998) had, in addition, children carrying DRPn with PFGE profiles which could not be found in children from any other center (Tables 1 to 3). Two of these lineages, characterized by PFGE types D and NNN, were each found in the same single DCC in consecutive years. Some clones such as D, QQ, RR, and BBB, although found in single centers each, were extensively spread within the DCC, providing evidence of persistence and cross-transmission.

Thirty-nine PFGE types (identified as "unique" in Tables 1 to 3) were obtained only once (single isolate) during the three surveillance periods.

TABLE 3. Diversity of microbiological profiles among DRPn isolated in DCCs in 1998

Penicillin resistance[a]	PFGE type	Capsular group/type	Antibiotype (resistant to[b]:)	Total no. of strains[c]:	No. of strains in DCC with clonal type											
					1	2	4	5	6	7	8	9	11	14	15	16
High	B	9	P, SXT, TX (7)	20	8					6					6	
	B	14	P, E (1), CC (1), TC (1), SXT (5), TX (3)	6		3									1	2
	MM	19	P, E, CC, TC, SXT	3											3	
	A	23	P, C, E (1), CC (1), TC, SXT, TX (18)	28	10	2				7	4				3	2
	Unique[d]	6	P, TC, SXT	1							1					
	Unique	19	P, E, CC, TC, SXT	1							1					
	nd[e]	nd	P, E, CC, TC, SXT	—[g]							1					
	nd	nd	P, C, TC, SXT	—												1
Low	E	6	P, E, CC, TC	4		4										
	M	6	P	1							1					
	BBB	6	P, E, CC, TC	10												10
	DDD	15	P, E, CC, TC (3)	7		4			2	1						
	H	19	P, E	1		1										
	RR	19	P, C, E (1), CC (1), TC, SXT	12												12
	FF	23	P	9				2	1	6						
	NNN	NT[f]	P, E, TC, SXT	3		3										
	Unique	19	P, TC (1), SXT	2						1					1	
	Unique	23	P	1							1					
	Unique	NT	P, E, CC, TC, SXT	3	1		1				1					
	nd	6	P, E, CC, TC, SXT	—												1
	nd	NT	P, E (1)	—	4											
	nd	NT	P, E, TC, SXT	—	1											
Susceptible	T	3	C (1), E, CC, TC	4				1			3					
	M	6	C (1), E (32), CC (32), TC, SXT (20)	35	1				1	8	18	3	3			1
	R	14	E, CC (1), TC (1), SXT (1)	27		11				11	4				1	
	H	19	E, CC, TC (3)	9	6		2					1				
	Unique	23	E, CC	1							1					
	Unique	11	C, E, CC	1			1									
	Unique	23	C	1								1				
	Unique	24	TC	1			1									
	nd	6	E, CC, TC, SXT (1)	—	1						1					
	nd	14	E	—						1						
	nd	19F	C, E, CC	—	1											
	nd	nd	E	—						1						
	nd	nd	E, CC, TC, SXT	—						1						

[a] See Table 1 for definitions of high and low resistance and susceptible.
[b] Drug abbreviations and parenthetical numbers are as defined for Table 1.
[c] The total number of strains was 191.
[d] Unique is as defined for Table 1.
[e] nd, not determined.
[f] NT, nontypeable.
[g] —, see definition in Table 1 footnotes.

Molecular typing of DSPn. The 89 DSPn recovered in DCCs 1, 2, and 7 during the first surveillance period that occurred in 1996 showed 26 distinct PFGE patterns (Table 5). Only two lineages, PFGE types h and z, were detected in two centers (DCCs 1 and 2). Together they accounted for 19 and 25% of the DSPn isolated in DCCs 1 and 2, respectively. However, the proportion of DSPn clones shared between DCCs 1, 2, and 7 was lower than the one observed for DRPn clones identified in the same DCCs (Table 5).

Interestingly, susceptible strains of PFGE type h had the same PFGE profile as the erythromycin-resistant clone H. The remaining 24 lineages identified among the DSPn were unique to each DCC. While most colonized only a few children, some clones, such as l, n, and y, were detected in several children.

DISCUSSION

Several studies have focused on the carriage of drug-resistant pneumococci by children attending DCCs (5, 6) and identification of risk factors associated with it (14, 20). However, detailed characterization of these isolates has relied mainly on the determination of capsular type only (9, 10, 14, 26). While these studies provide important information for local and international surveillance and may form the basis for the design of conjugate vaccines to be introduced in the near future, a better understanding of the dynamics of pneumococcal population in these settings requires the use of more discriminative techniques such as molecular typing (11, 13, 18, 28). In this study we used PFGE, the "gold standard" for molecular fingerprinting (27), to characterize a large collection of *S. pneumoniae* isolates recovered from the nasopharynges of healthy children attending several DCCs in Lisbon in three consecutive yearly 2-month surveillance periods. Overall, 413 DRPn isolates were characterized by antibiotype, serotype, and PFGE type and 89 DSPn were also molecularly typed. To our best knowledge, the Lisbon DCC surveillance study described here represents the single largest surveillance effort carried out in DCCs in which molecular techniques were systematically applied. We characterized not only penicillin-insensitive isolates

TABLE 4. Proportion of epidemic clones of DRPn in each DCC

Yr and DCC	No. of DRPn fully characterized[a]	No. of clonal types	Epidemic clones (PFGE types)	% of children carrying DRPn colonized by clones:	
				A + B[b]	E + H + M + R + DDD[c]
1996					
1	35	10	**B**, H, M, R	20	40
2	4	2	**B**, E	25	75
3	7	4	**A**, M	57	14
4	3	3	**A**, **B**	67	0
5	2	2		0	0
6	5	3	**A**, H	60	20
7	35	9	**A**, **B**, E, H, M	37	23
Total	91			33	30
1997					
1	27	6	**A**, H, M, (FF)[d]	26	30
2	28	7	E, M	0	50
4	9	4	H, DDD	0	78
6	10	2	H	0	30
7	26	5	**A**, E, M, (FF)	19	8
8	15	8	**A**, **B**, M, DDD	33	27
9	4	3	M, (FF)	0	50
10	0	0		0	0
11	6	2	**A**, M	83	17
12	3	3	M	0	33
13	3	3	**B**	33	0
14	0	0		0	0
Total	131			18	32
1998					
1	31	5	**A**, **B**, H, M	58	23
2	23	6	**A**, **B**, E, H, R, DDD	22	87
4	5	4	H	0	40
5	3	2	(FF)	0	0
6	4	3	M, (FF), DDD	0	75
7	40	7	**A**, **B**, M, R, (FF), DDD	33	50
8	35	9	**A**, M, R	11	66
9	5	3	H, M	0	80
11	3	1	M	0	100
14	0	0		0	0
15	15	5	**A**, **B**, R	67	7
16	27	5	**A**, **B**, M	15	4
Total	191			28	44

[a] Antibiotype, serotype and PFGE type determined.
[b] Isolates with PFGE types A and B (in boldface in previous column) are resistant to penicillin and typically susceptible to macrolides.
[c] Isolates with PFGE types E, H, M, R, and DDD are typically resistant to macrolides and are susceptible or have low-level resistance to penicillin.
[d] Clone FF is a recurrent clone that has low-level resistance to penicillin and that is susceptible to other antimicrobial agents.

but also all other drug-resistant pneumococci. In addition, we included a group of isolates susceptible to all antimicrobial agents, which allowed comparisons between the genetic diversity of these pneumococcal populations. The observations described in this study allow several conclusions.

Increase in the frequency of DRPn colonizing children in DCCs. The frequency of DRPn increased approximately 5% per year, reaching 50% in the 1998 sampling. However, changes in the rates of resistance to the antimicrobial agents tested were not uniform. Rates of resistance to chloramphenicol, and ceftriaxone remained steady in all periods and below 10%. Resistance to erythromycin and tetracycline did not increase from the first to the second surveillance period and then increased 5%, reaching 26% of all pneumococci, in 1998. Clindamycin resistance increased from 14% in 1996 to 18% in 1997 and then remained steady.

Penicillin resistance increased from 20% in 1996 to 26% in 1998 with a peak of 36% in 1997. Interestingly, proportions of pneumococcal isolates with high-level resistance to penicillin (MIC $\geq$ 0.5 μg/ml) did not follow this trend; they were 11, 8, and 13% in the consecutive samples. On the other hand, low-level resistance (MIC, $\geq$0.1 and $<$0.5 μg/ml) was detected in 9, 22, and 13% of the pneumococci in 1996, 1997, and 1998, respectively. The reason for the proliferation of strains with low-level resistance to penicillin in the surveillance in 1997 is not known. Data obtained at the time of the sampling concerning antibiotic consumption habits and illnesses did not reveal significant differences between the three surveillance periods (data not shown). Antibiotic consumption was high, with similar values in all sampling periods, around 30%, and the majority of antibiotics taken were β-lactams (around 70%) followed by macrolides (18%).

From the data obtained by molecular typing two processes seem to be contributing to the increase in the frequency of DRPn: cross-transmission of resistant bacteria and dissemination of resistance determinants through recombinational mechanisms.

Spread of drug resistance by cross-transmission of DRPn clones. The frequent isolation of DRPn sharing the same antibiotype, serotype, and PFGE type from several children indicates that cross-transmission plays an important role in the dissemination of DRPn. Eight specific lineages, including two internationally spread penicillin-resistant clones, five macrolide-resistant lineages, and a clone expressing only low-level penicillin resistance, appear to be particularly successful in this process.

(i) Internationally disseminated penicillin-resistant clones. A total of 119 highly penicillin-resistant pneumococci were isolated from the nasopharynges of children during the three

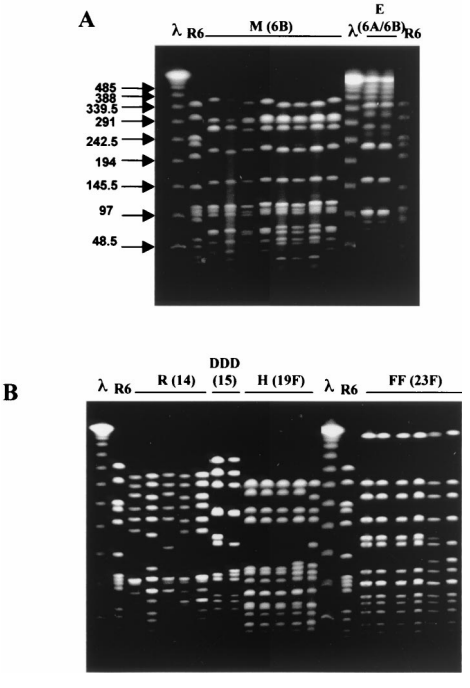

FIG. 1. PFGE profiles of drug-resistant *S. pneumoniae* epidemic clones with low-level resistance or susceptibility to penicillin isolated from children attending various DCCs in Lisbon, Portugal. (A) PFGE types M and E; (B) PFGE types R, DDD, H, and FF. Capsular types of the isolates are in parentheses. The lambda ladder and R6 are indicated and were used as molecular weight markers. Numbers at the left side of the gel show molecular sizes in kilobases.

TABLE 5. Diversity of genetic backgrounds (PFGE types) of DSPn isolates compared to that of DRPn isolates in DCCs 1, 2, and 7

Type of pneumococci	DCC	No. of pneumococcal carriers[b]	PFGE types (no. of isolates with PFGE type)[a]	No. of clonal types shared between DCCs 1, 2, and 7	% of pneumococcal carriers with clonal types shared between DCCs 1, 2, and 7
DSPn	1	31	**h** (1), m (6), n (13), o (4), p (1), q (1), **z** (5),	2	19
	2	32	**h** (6), r (5), s (2), t (1), u (1), v (1), w (2), x (1), y (8), **z** (2), nd[b] (3)	2	25
	7	32	a (5), b (1), c (2), d (3), e (1), f (4), g (1), i (2), j (1), k (1), l (8), nd (3)	0	0
	Total	95			
DRPn	1	37	**B** (7), **H** (4), I (1), J (1), K (1), L (8), **M** (12), R (1), Z (1), AA (1)	3	62
	2	5	**B** (1), **E** (3), nd (1)	2	80
	7	35	A (12), **B** (1), D (11), **E** (2), G (1), **H** (1), **M** (5), DD (1), XX (1)	4	26
	Total	77			

[a] PFGE types in boldface are those shared between DCCs 1, 2, and 7. There were a total of 26 DSPn PFGE types and a total of 16 DRPn PFGE types.
[b] nd, not determined.

periods. Of these, as many as 107 (90%) were represented by isolates belonging to the two internationally spread Spanish/U.S. (serotype 23F, PFGE type A) and French/Spanish (serotype 9V or 14, PFGE type B) clones. This finding supports the notion that the nasopharynges of children in DCCs are a major reservoir of disease-causing drug-resistant clones of pneumococci. Over the three surveillance periods, the frequency of these two pandemic clones (and essentially also the frequency of all high-level penicillin-resistant pneumococci) has remained between 28 and 33% of all DRPn with a decrease to 18% in 1997 when only two isolates of the French/Spanish clone were detected.

(ii) Macrolide-resistant lineages. In contrast to the highly penicillin-resistant international clones, five erythromycin-resistant lineages, most frequently unaccompanied by resistance to penicillin, have been isolated with increasing frequency during the same surveillance periods. These clones (PFGE types M, E, R, DDD, and H) were repeatedly isolated from more than one DCC and during more than one study period suggesting "epidemic" nature. Together, isolates belonging to these five lineages accounted for 31% (28 of 91) of the DRPn isolates obtained in 1996 (8), 32% (42 of 131) of those obtained in 1997, and 44% (84 of 191) of those obtained in 1998.

(iii) A lineage expressing only low-level penicillin resistance. Apart from the seven clones just described, clone FF with low-level resistance to penicillin was also recurrent, persistent, and disseminated in more than one DCC. When the frequencies of these eight clones are added, it seems that the majority of DRPn colonizing the nasopharynges of children were members of eight intraspread clones which through the years of 1996, 1997, and 1998 represented 64 (58 of 91 strains), 60 (79 of 131 strains), and 77% (147 of 191 strains) of all DRPn.

Dissemination of resistance determinants among pneumococci colonizing the nasopharynx. The high number of DRPn isolates with different clonal types but carrying the same resistance traits, such as resistance to macrolides, lincosamides, and tetracycline, suggests that horizontal transfer of resistance genes may be a common genetic event among colonizing isolates. The identification of isolates sharing a common PFGE profile but carrying different resistant determinants and various levels of resistance to penicillin (as observed, for example, in lineage H and its fully susceptible counterpart, h) corroborates this hypothesis. The nasopharynges of children attending DCCs where cross-transmission of pathogens is a frequent event may well be a suitable ecological niche for the emergence of resistance in several different genetic backgrounds.

DCCs are autonomous epidemiological units. The most interesting conclusion that emerges from these studies is the apparent uniqueness of the microbiological profile of each DCC. Two types of DRPn contribute to these profiles: (i) a relatively few (eight) epidemic clones which nevertheless colonize many children and (ii) a large number of distinct clonal types (45 of the 57 total PFGE types) which are confined each to a single DCC and which are primarily responsible for generating the unique microbiologcial floras of each DCC. Even higher diversity and less sharing of clonal types is observed among the DSPn isolates. These findings suggest that each DCC may be considered an autonomous epidemiological unit suitable for testing the efficacies of various intervention strategies designed to decrease the level of antimicrobial resistance and cross-transmission of *S. pneumoniae* that colonizes children attending DCCs in Lisbon.

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