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High Rates of Transmission of and Colonization by *Streptococcus pneumoniae* and *Haemophilus influenzae* within a Day Care Center Revealed in a Longitudinal Study▽

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Day care centers (DCCs) are unique settings where young children are at increased risk for colonization by pneumococci and *Haemophilus influenzae*. Although point prevalence studies in DCCs are frequent, only a few longitudinal studies on the dynamics of colonization have been published. We conducted a 1-year longitudinal study with 11 sampling periods on nasopharyngeal carriage of pneumococci and *H. influenzae* among 47 children who attended a single DCC. All isolates were antibiotyped and genotyped by pulsed-field gel electrophoresis. Pneumococci were also serotyped. Of the 414 samples obtained, 61.4% contained pneumococci, and 87% contained *H. influenzae*. Only 8.3% of the samples were negative for both species. Twenty-one pneumococcal clones and 47 *H. influenzae* clones were identified. Introduction of clones occurred during all year. Ninety-eight percent and 96% of all pneumococcal and *H. influenzae* isolates, respectively, belonged to clones shared by more than one child. Children were sequentially colonized with up to six pneumococcal clones (mean, 3.6) and five serotypes and nine *H. influenzae* clones (mean, 7.1). Clones with increased capacity for transmission and/or prolonged colonization were identified in both species. These two fitness properties appeared to be independent. In conclusion, among DCC attendees, a high rate of acquisition and turnover of strains was observed, and all children were overwhelmingly colonized by clones shared with others. DCCs are units where permanent introduction of new clones occurs, and attendees, as a whole, provide a pool of hosts where the fittest clones find privileged opportunities to persist and expand.

Studies conducted during the last decade have highlighted the important role of day care centers (DCCs) as unique places where young children with immature immune systems and poor hygienic behavior are crowded together, resulting in an increased risk for colonization and transmission of upper respiratory tract pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* (1, 4, 5, 10, 23, 30).

While point prevalence studies in DCCs to study colonization by these bacteria have been conducted in several countries (reviewed in reference 7), longitudinal studies are less frequent and have often focused on the individual host and not on a particular epidemiological setting (9, 14, 16). By looking at the DCC as a unit, one would expect to obtain additional information on the fitness capacities (for transmission and persistence) of individual clones, as they would be exposed to the same pool of hosts (the attendees). To our best knowledge, extended longitudinal studies that have systematically applied genotyping techniques to study pneumococci and *H. influenzae* in DCCs with such objectives in mind are very scarce. Trottier et al. studied *H. influenzae* colonization among 38 DCC attendees for 4 months (29), and Yagupsy et al. conducted a 7-month study focusing on the transmission of drug-resistant pneumococci among 48 children from two DCCs (30). A third study by Raymond et al. followed 53 children in an orphanage over 1 year and described the colonization patterns of pneumococci and *H. influenzae* (19, 20). However, as one might expect, this latter setting was more isolated than the DCC, and thus its dynamics were peculiar.

In Portugal, according to the National Statistics Institute, more than 70% of preschool children attend DCCs (15). Previous point prevalence studies conducted among Portuguese DCC attendees have found high pneumococcal and *H. influenzae* colonization rates (over 70%) (5, 12) with abundant representation of international clones (22).

To understand the dynamics of colonization that led to such high colonization rates, we conducted a 1-year longitudinal study among a group of 47 children attending a single DCC where all pneumococci and *H. influenzae* isolates were genotyped.

MATERIALS AND METHODS

Study population. Forty-seven children (24 females and 23 males) attending a DCC in Lisbon, Portugal, were enrolled. In the beginning of the study, the

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children's ages ranged from 14 to 37 months (mean, 24.3 months). At the DCC the children were divided into three rooms (here, designated I, II, and III; children are identified by these room designations); however, they were often together in the playground.

Approval for the study was obtained from the Ministry of Education and the DCC director. Signed informed consent was obtained from the parents or guardians of all children.

Questionnaires. Information on the children's illnesses and antimicrobial consumption in the month preceding each sampling was obtained through questionnaires filled in by the parents. Children's physicians were not interviewed, nor were the children observed by the pediatricians of our team. Therefore, the information obtained was used only as an indicator of the health status of the group.

Sampling period and sampling. Nasopharyngeal samples were taken 11 times, 3 to 9 weeks apart from February 1998 through February 1999 (i.e., in the pre-pneumococcal conjugate vaccine era). Sampling occurred in weeks 1, 5, 11, 15, 20, 29, 33, 38, 42, 47, and 50. The longer interruption between weeks 20 and 29 was due to summer holidays, during which this DCC was closed. Samples were taken by a pediatric nurse using calcium alginate swabs, as previously described (17).

Isolation of pneumococci and *H. influenzae*. Swabs were inoculated into culture medium within 4 h. Pneumococci and *H. influenzae* were selectively cultured in blood agar with gentamicin and chocolate blood agar containing Iso-Vitalex and bacitracin, respectively (21). Pneumococcal presumptive identification was based on α-hemolysis and optochin susceptibility; *H. influenzae* isolates were identified based on the requirement of X and V factors for growth. Routinely, a single colony of each species was isolated, cultured, and frozen.

Antimicrobial susceptibility testing. The Kirby-Bauer technique was used according to the Clinical Laboratory Standards Institute recommendations and definitions (2). Susceptibility to erythromycin, clindamycin, tetracycline, chloramphenicol, and sulfamethoxazole-trimethoprim (SXT) was determined for isolates of *S. pneumoniae*, and susceptibility to ampicillin, amoxicillin-clavulanic acid, cefuroxime, erythromycin, azithromycin, and SXT was determined for isolates of *H. influenzae.* Antibiotic discs were purchased from Oxoid (Hampshire, England). In addition, the MIC of penicillin G was measured for pneumococci using Etest (AB Biodisk, Solna, Sweden), and *H. influenzae* isolates were tested for the presence of β-lactamase (DrySlide; Difco Laboratories, Detroit, MI).

Serotyping. Pneumococcal strains were serotyped by the quellung reaction using commercially available antiserum (Statens Serum Institut, Copenhagen, Denmark). *H. influenzae* strains were not serotyped.

PFGE. Pneumococcal and *H. influenzae* total DNA were prepared as previously described (22). After restriction with SmaI, DNA fragments were separated by pulsed-field gel electrophoresis (PFGE) (22). Patterns were assigned by visual inspection of the profiles, using currently accepted criteria (28).

Duration of carriage and reacquisition. Duration of carriage was calculated from the date of the first isolation of a strain to the date of its last isolation, provided that it was not detected in the next two consecutive samples. Reacquisition of a strain was defined as its reisolation (from a child previously known to carry it) after being undetected for at least two consecutive samples. The ratio of the number of isolates of a clone to the number of children colonized (I/CC) was used as an indirect measure of the capacity to induce persistent colonization.

RESULTS

Nasopharyngeal samples and questionnaires. Of the 517 expected samples, 414 (80%) were obtained. The difference resulted mainly from the absences of children from the DCC when the sampling occurred. Participation varied from 63.8% to 93.6%. The mean number of samples per child was 8.8, ranging from a minimum of 5 (two children) to all 11 samples (nine children, or 19%).

For the reasons explained above, questionnaires were used only as an indicator of the global health status of the population under study and should be interpreted with caution. Fifty-five percent of the questionnaires indicated that the child had been healthy within the month preceding the sampling and had not taken any medicine; the remaining questionnaires indicated that within that period the child had at least once taken some medication. The symptoms leading to medication most often reported resembled, in the vast majority, respiratory tract infections of viral origin. Otitis media was reported in 8% of the questionnaires, and sore throat was reported in 5%. An outbreak of chickenpox occurred in May 1998. Antimicrobial use was reported in 18% of the questionnaires and was mainly associated with diagnosis of otitis media, sore throat, and respiratory tract infections.

Nasopharyngeal colonization and antimicrobial resistance. A total of 61.4% of the samples contained pneumococci, with rates ranging from 38% to 77% according to the sampling period. Similarly, 87.0% were positive for *H. influenza*e (range, 74% to 97%). Overall, 56.7% of the samples contained both microorganisms, and 8.3% had none.

Simultaneous colonization by two or more strains of the same species was not thoroughly investigated but was detected in three samples that contained two pneumococcal strains.

Out of the 257 pneumococcal isolates, 35% were susceptible to all antimicrobial agents tested, and 24.5% had decreased susceptibility to penicillin. Other resistance rates were 52% to erythromycin, 49% to clindamycin, 35% to tetracycline, 24% to SXT, and 8% to chloramphenicol. Over time, resistance rates to penicillin and macrolides ranged from 0% to 53% and from 39% to 58%, respectively. Rates of resistance to one or more of the antimicrobials tested ranged from 48% to 100%.

Out of the 360 *H. influenzae* isolates, 80% were susceptible to all antimicrobial agents tested, 12% were resistant to ampicillin due to β-lactamase production, and 8% were resistant to SXT. β-Lactamase nonproducing ampicillin-resistant *H. influenzae* strains were not detected. Resistance rates to ampicillin ranged from 0 to 47%, according to the sampling period.

Pneumococcal serotypes. Thirteen serotypes were identified which were, in decreasing order of abundance, 19F (34.2%), 23F (15.2%), 6B (11.3%), 14 (8.6%), 10A (7.8%), 19A (5.4%), 9V (3.1%), 11A (1.9%), 16F (1.9%), 18F (1.9%), 15A (0.8%), 8 (0.4%), and 23B (0.4%). Seven percent of the isolates were nontypeable (NT).

Patterns of pneumococcal colonization. All but one child was colonized by pneumococci at least once during the study (the number of positive samples per child ranged from 1 to 10): 6% (children I-h, I-l, and II-i) were always colonized (all samples were positive), and 91% were intermittently colonized (Table 1). Among the latter group, four children (I-e, II-m, III-e, and III-l) were very frequently colonized, and four children (I-c, I-d, III-b, and III-p) were rarely colonized. A single child (III-h) who was never positive for pneumococcus infection was sampled eight times and was found to have *H. influenzae* on seven occasions, suggesting that incorrect sampling did not occur.

Based on serotype and PFGE analysis, we observed that for any pair of two consecutive samples from the same child, 39.1% (144 of 368 pairs) of the cases corresponded to a transition between pneumococcal isolation and no isolation (or vice versa), 25.3% (93 of 368) contained two different clones, 17.1% (63 of 368) contained the same clone, and 18.5% (68 of 368) yielded no pneumococci isolated in either sample.

Among the 45 children who had two or more pneumococcus-positive samples, 93% were colonized by more than one clone (Table 1). The number of pneumococcal serotypes and clones detected per child ranged from one to five and from one

TABLE 1. Summary of colonization patterns

Child	Total no. of samples	*S. pneumoniae* colonization					*H. influenzae* colonization			
		No. of positive samples	No. of serotypes	No. of clones	Maximum period of carriage (wks)[a]	No. of reacquisitions	No. of positive samples	No. of clones	Maximum period of carriage (wks)[a]	No. of reacquisitions
I-a	10	4	3	3	NA	1	8	8	NA	0
I-b	9	6	4	4	3	1	9	8	5	0
I-c	5	1	1	1	NA	0	5	3	6	1
I-d	9	2	1	1	4	0	6	4	8	0
I-e	8	7	2	2	24	1	6	4	NA	1
I-f	9	7	1	1	42	0	8	7	4	0
I-g	10	4	3[b]	4	NA	0	9	3	9	0
I-h	7	7	5	5	13	1	7	5	20	0
I-i	8	4	2	2	14	1	6	3	14	1
I-j	8	6	3[b]	4	13	0	8	6	4	1
I-k	8	6	3	3	14	0	8	5	4	0
I-l	7	7	2	2	32	0	6	4	10	0
I-m	5	4	2	2	13	0	4	3	3	0
I-n	11	6	4	5	13	0	10	7	10	1
I-o	11	7	4	4	9	1	8	7	4	1
I-p	11	9	4	4	22	0	8	6	5	0
II-a	9	6	3	3	28	0	9	8	4	0
II-b	6	4	2	2	22	0	6	5	22	0
II-c	9	5	2	2	31	0	9	7	14	0
II-d	7	3	2	2	14	0	5	5	NA	0
II-e	6	5	3	3	18	0	6	6	NA	0
II-f	10	8	2	2	24	1	9	7	13	0
II-g	10	6	2	2	18	1	9	5	9	1
II-h	8	5	2	2	17	0	8	6	12	0
II-i	6	6	4[b]	5	NA	1	6	6	NA	0
II-j	9	6	4	6	9	0	9	6	13	0
II-k	8	3	2	2	13	0	5	5	NA	0
II-l	11	7	3	3	18	0	11	9	13	0
II-m	10	9	3[b]	5	15	0	9	8	NA	1
II-n	8	4	3[b]	4	NA	0	6	5	4	0
II-o	7	2	2	2	NA	0	7	7	NA	0
III-a	10	5	3	3	9	1	8	6	18	0
III-b	9	2	2	2	NA	0	7	5	28	0
III-c	10	6	1	1	45	0	10	6	9	2
III-d	11	7	4	4	13	0	10	6	17	0
III-e	11	10	5[b]	6	13	1	10	8	13	0
III-f	9	5	3	3	5	0	8	6	18	0
III-g	9	7	2	3	9	0	6	4	6	0
III-h	8	0	0	0	0	0	7	7	NA	0
III-i	11	7	2[b]	4	8	0	9	5	17	0
III-j	10	5	3	3	8	0	9	5	14	1
III-k	11	8	2[b]	3	30	0	9	7	4	0
III-l	11	10	3	3	9	1	10	5	14	0
III-m	7	4	3	3	3	0	5	4	4	0
III-n	7	7	2	2	33	0	7	4	14	0
III-o	10	3	2	2	3	0	8	5	8	0
III-p	10	2	1	1	NA	1	7	6	5	0

[a] NA, not applicable as no two identical consecutive samples were detected.
[b] NT strains were also detected.

to six, respectively. Globally, 135 child-clones (defined as the total sum of the number of clones isolated from each individual child) were obtained among the 414 samples (80% of the total anticipated number of samples), which led us to calculate that the 47 children were colonized by a mean of 3.6 pneumococcal clones during the 1-year study. Similarly, 129 child-serotypes were obtained, which led us to estimate that children were colonized by a mean of 3.4 serotypes.

Prolonged colonization was observed in 23% of the children: children I-f and III-c were colonized by the same strain for more than 9 months, and nine other children were also colonized by periods of time that ranged from 5 to 7 months (Tables 1 and 2).

Reacquisition of a clone (and of the corresponding serotype) appeared to have occurred in 28% of the children. In addition, five events of serotype reacquisition (but of different strains) were detected: child III-g (serotype 6B), children II-j and II-m (23F), and children II-m and III-I (NT).

Patterns of *H. influenzae* colonization. All 47 children were colonized by *H. influenzae* at some time during the study (the number of positive samples per child ranged from 4 to 11), and 34% of the children were persistently colonized.

TABLE 2. Detailed genotypes of *S. pneumoniae* per child

Room and child	Clone detected at week[a]:										
	1	5	11	15	20	29	33	38	42	47	50
Room I											
I-a	Neg	19F-Pn3	10A-Pn7		Neg	Neg	Neg	19F-Pn3	Neg	23F-Pn14	Neg
I-b	23F-Pn1	Neg		10A-Pn7	19F-Pn3	Neg	Neg	19F-Pn3		6B-Pn9	6B-Pn9
I-c		Neg	19F-Pn3				Neg	Neg			Neg
I-d	Neg		19F-Pn3	19F-Pn3	Neg	Neg	Neg		Neg	Neg	Neg
I-e	19F-Pn3	11A-Pn8		11A-Pn8	11A-Pn8	11A-Pn8	Neg		19F-Pn3	19F-Pn3	
I-f	Neg	19F-Pn3	19F-Pn3		19F-Pn3	19F-Pn3	19F-Pn3	19F-Pn3		19F-Pn3	Neg
I-g	11A-Pn8	Neg	Neg	10A-Pn7	NT-Pn10	Neg		Neg	Neg	Neg	6B-Pn6
I-h	9V-Pn2	10A-Pn7	16F-Pn13		19A-Pn11		19A-Pn11	16F-Pn13		Neg	23F-Pn14
I-i	Neg	Neg		19F-Pn3		19F-Pn3	19A-Pn11	Neg	19F-Pn3	Neg	
I-j		NT-Pn5	NT-Pn5		10A-Pn7		10A-Pn7	Neg	Neg	19F-Pn3	14-Pn2-R
I-k	9V-Pn2	9V-Pn2	9V-Pn2	9V-Pn2				Neg	Neg	19A-Pn11	18F-Pn4
I-l	19F-Pn3	19F-Pn3	19F-Pn3	19F-Pn3		19F-Pn3	19F-Pn3			19A-Pn11	
I-m					19F-Pn3	Neg	19F-Pn3			23F-Pn14	23F-Pn14
I-n	Neg	Neg	Neg	NT-Pn5	6B-Pn12	19F-Pn3	6B-Pn12	Neg	Neg	23F-Pn14	8-Pn15
I-o	Neg	Neg	19F-Pn3	19F-Pn3	9V-Pn2	9V-Pn2	19F-Pn3	Neg	19A-Pn11	Neg	18F-Pn4
I-p	6B-Pn12	10A-Pn7	19F-Pn3	19F-Pn3	19F-Pn3	19F-Pn3	19F-Pn3	Neg	Neg	15A-Pn15	15A-Pn15
Room II											
II-a	19F-Pn3	19F-Pn3		19F-Pn3	Neg	19F-Pn3		Neg	23F-Pn14	Neg	14-Pn2-R
II-b			19F-Pn3			19F-Pn3	19F-Pn3	Neg		23F-Pn14	Neg
II-c	23F-Pn1	Neg	19F-Pn3		19F-Pn3		Neg	19F-Pn3	19F-Pn3	Neg	Neg
II-d	Neg	Neg		23F-Pn1		23F-Pn1		19A-Pn11	Neg	Neg	
II-e			19F-Pn3			19F-Pn3	Neg	23F-Pn14		23F-Pn14	14-Pn2-R
II-f	19F-Pn3	23F-Pn1	23F-Pn1	23F-Pn1	23F-Pn1	23F-Pn1	Neg	19F-Pn3		19F-Pn3	Neg
II-g	Neg	Neg	23F-Pn1	23F-Pn1	23F-Pn1	23F-Pn1	Neg	Neg	23F-Pn1	18F-Pn4	
II-h	Neg		19F-Pn3		19F-Pn3		23F-Pn14	Neg	23F-Pn14	Neg	23F-Pn14
II-i	19F-Pn3	NT-Pn5		10A-Pn7			19F-Pn3			23F-Pn1	14-Pn2-R
II-j	23F-Pn1		10A-Pn7	Neg	19F-Pn3	19F-Pn3	Neg	Neg		23F-Pn14	14-Pn2-R
II-k	Neg		Neg		23F-Pn1	Neg	23F-Pn1	Neg	19F-Pn3	Neg	
II-l	Neg	Neg	10A-Pn7	19A-Pn11	19A-Pn11	19A-Pn11	19A-Pn11	Neg	Neg	14-Pn2-R	14-Pn2-R
II-m	NT-Pn17	23F-Pn1	23F-Pn1		23F-Pn1, NT-Pn5	19A-Pn11	19A-Pn11	Neg	23F-Pn14	14-Pn2-R	14-Pn2-R
II-n	19F-Pn3	NT-Pn5	Neg				Neg	Neg	23F-Pn1	Neg	18F-Pn4
II-o	Neg	Neg	10A-Pn7			Neg	Neg			18F-Pn4	Neg
Room III											
III-a	Neg		6B-Pn9	Neg	6B-Pn9	Neg	19A-Pn11	6B-Pn9	Neg	14-Pn2-S	Neg
III-b	Neg	Neg	Neg		10A-Pn7		19F-Pn3	Neg	Neg	Neg	Neg
III-c	Neg	19F-Pn3	Neg	19F-Pn3	Neg	19F-Pn3	19F-Pn3	19F-Pn3	Neg		19F-Pn3
III-d	Neg	Neg	Neg	9V-Pn2	19F-Pn3	Neg	19F-Pn3	10A-Pn7	6B-Pn6	6B-Pn6	6B-Pn6
III-e	NT-Pn5	10A-Pn7	Neg	10A-Pn7	19F-Pn3	19F-Pn3	19F-Pn3	6B-Pn9	NT-Pn5	6B-Pn9	14-Pn2-S
III-f	Neg	Neg		10A-Pn7	10A-Pn7		19F-Pn3	Neg	6B-Pn6	6B-Pn6	Neg
III-g	Neg	6B-Pn9	Neg			6B-Pn6	6B-Pn6	6B-Pn6	23F-Pn1	23F-Pn1	23F-Pn1
III-h	Neg	Neg		Neg	Neg	Neg		Neg	Neg	Neg	Neg
III-i	Neg	Neg	Neg	NT-Pn18	6B-Pn9	NT-Pn5	NT-Pn5	Neg	14-Pn2-S	14-Pn2-S	14-Pn2-S
III-j		Neg	23B-Pn16	Neg	Neg	Neg	Neg	6B-Pn9	14-Pn2-S	14-Pn2-S	14-Pn2-S
III-k	Neg	Neg	16F-Pn13	Neg	NT-Pn5, 16F-Pn13	NT-Pn5	NT-Pn5, 16F-Pn13	14-Pn2-S	NT-Pn5	14-Pn2-S	NT-Pn5
III-l	Neg	19F-Pn3	19F-Pn3	10A-Pn7	10A-Pn7	19F-Pn3	19F-Pn3	19F-Pn3	6B-Pn9	6B-Pn9	6B-Pn9
III-m					Neg	19F-Pn3	Neg	10A-Pn7	Neg	6B-Pn6	6B-Pn6
III-n		19F-Pn3	19F-Pn3	19F-Pn3	19F-Pn3		19F-Pn3	14-Pn2-S		14-Pn2-S	
III-o	Neg	Neg	Neg	Neg	Neg		Neg	14-Pn2-S	Neg	6B-Pn9	6B-Pn9
III-p	Neg	19F-Pn3	Neg	Neg	Neg	Neg	19F-Pn3	Neg	Neg	Neg	

[a] Neg, no pneumococci detected; blank cell, not sampled; S, susceptible to penicillin; R, resistant to penicillin.

Based on PFGE analysis, we observed that colonization by *H. influenzae* was quite dynamic: for any pair of two consecutive samples from the same child, the majority (59.9%, or 215 of 359 pairs) contained two different clones, and in 21.2% of the cases a transition between colonization and no colonization (or vice versa) seemed to have occurred. In a minority of the cases, the consecutive isolation of the same clone occurred (16.4%), and in 2.5% of the cases, the two consecutive samples did not contain *H. influenzae*.

A high strain turnover rate was observed in seven children (15%) from whom all positive *H. influenzae* samples contained different clones (e.g., children I-a, II-o, and III-h) (Tables 1 and 3). By contrast, long colonization periods of 4 to 6 months were observed for seven children (e.g., children II-b, III-b, and III-d).

The number of *H. influenzae* clones detected per child ranged from three to nine Globally, 267 child-clones (defined as the total sum of the number of clones isolated from each individual child) were obtained among the 414 samples (80% of the total anticipated number of samples), which led us to calculate that the 47 children were colonized by a mean of 7.1 *H. influenzae* clones during the 1-year study. In 19% of the children, reacquisition of a clone appeared to have occurred (Table 1).

Pneumococcal clones. Twenty-one pneumococcal clones were detected (Tables 2 and 4; Fig. 1), of which three were detected all year and among children from all three rooms: 19F-Pn3 (i.e., a serotype 19F clone with pneumococcus [Pn] PFGE pattern 3; named Portugal^{19F} ST177 by the Pneumococcal Molecular Epidemiology Network [13]), 23F-Pn1

TABLE 3. Detailed genotypes of *H. influenzae* per child

Room and child	Clone detected at week[a]:										
	1	5	11	15	20	29	33	38	42	47	50
Room I											
I-a	Hi-12	Hi-9	Hi-16		Hi-3	Neg	Hi-11	Unique	Hi-7	Neg	Unique
I-b	Hi-12	Hi-9		Hi-28	Hi-35	Hi-30	Hi-11	Hi-11		Hi-47	Hi-1
I-c		Hi-13	Hi-13				Hi-13	Hi-9			Hi-8
I-d	Hi-14		Neg	Neg	Hi-31	Unique	Neg		Hi-7	Hi-7	Hi-7
I-e	Hi-10	Neg		Hi-26	Hi-10	Unique	ND		Hi-13	Neg	
I-f	Hi-12	Hi-19	Hi-10		Hi-35	Hi-11	Hi-11	Hi-9		Hi-5	Neg
I-g	Hi-14	Hi-14	Hi-17	Hi-17	Hi-17	ND		ND	ND	Hi-5	Hi-5
I-h	Hi-10	Hi-20	Hi-10		Hi-10		Hi-3	Hi-13			Hi-7
I-i	Hi-13	Unique		Hi-13		Hi-35	Hi-35	Hi-13	Neg	Neg	
I-j		Hi-20	Hi-10		Hi-13		Hi-9	Hi-13	Hi-13	Hi-47	Hi-3
I-k	Hi-12	Hi-12	Hi-10	Hi-10				Hi-13	Hi-43	Hi-3	Hi-3
I-l	Hi-13	Hi-13	Hi-13	Hi-27		Hi-11	Neg			Hi-7	
I-m					Hi-35	Neg	Hi-9			Hi-7	Hi-7
I-n	Hi-12	Hi-9	Hi-9	Hi-9	Hi-13	Hi-17	Hi-35	Hi-13	Neg	Hi-5	Hi-11
I-o	Hi-9	Hi-12	Hi-15	Hi-27	Neg	Neg	Hi-43	Hi-7	Hi-7	Hi-43	Neg
I-p	Hi-9	Hi-48	Hi-26	Hi-27	Hi-27	Neg	Neg	Neg	Hi-44	Hi-7	Hi-7
Room II											
II-a	Hi-10	Hi-48		Hi-27	Unique	Hi-11		Hi-7	Hi-7	Hi-3	Hi-8
II-b			Hi-13			Unique	Hi-13	Hi-7		Hi-38	Hi-3
II-c	Hi-12	Hi-48	Hi-15		Hi-35		Hi-44	Hi-44	Hi-7	Hi-44	Hi-8
II-d	Hi-12	Unique		Hi-19		Hi-13		Hi-17	Neg	Neg	
II-e			Hi-13			Hi-27	Hi-35	Hi-7		Hi-3	Hi-8
II-f	Hi-10	Hi-10	Unique	Hi-27	Hi-13	Hi-32	Hi-13	Hi-7		Neg	Hi-3
II-g	Hi-12	Neg	Hi-13	Hi-48	Hi-27	Hi-13	Hi-13	Hi-18	Hi-18	Hi-18	
II-h	Hi-12		Hi-19		Hi-48		Hi-3	Hi-7	Hi-7	Hi-38	Hi-7
II-i	Hi-10	Unique		Hi-27			Hi-44			Hi-7	Hi-3
II-j	Hi-14		Hi-9	Hi-27	Hi-13	Hi-13	Hi-13	Hi-7		Hi-3	Hi-3
II-k	Hi-12		Hi-48		Neg	Neg	Hi-7	Hi-13	Neg	Hi-3	
II- l	Hi-10	Hi-48	Hi-27	Hi-31	Hi-13	Hi-7	Hi-13	Hi-7	Hi-26	Hi-3	Hi-9
II-m	Hi-12	Hi-19	Hi-13		Hi-27	Hi-32	Hi-13	Neg	Hi-7	Hi-3	Hi-8
II-n	Hi-12	Neg	Neg				Hi-13	Hi-7	Hi-7	Hi-9	Hi-8
II-o	Hi-12	Hi-48	Hi-27			Hi-13	Hi-32			Hi-7	Hi-9
Room III											
III-a	Hi-10		Hi-41	Hi-32	Hi-30	Hi-5	Neg	Hi-5	Hi-9	Hi-5	Neg
III-b	Neg	Hi-10	Hi-10		Hi-33		Hi-10	Hi-13	Hi-5	Hi-18	Neg
III-c	Hi-10	Hi-9	Hi-28	Hi-10	Hi-28	Unique	Hi-10	Hi-5	Hi-3		Hi-5
III-d	Hi-15	Unique	Hi-27	Hi-27	Hi-1	Neg	Hi-5	Hi-5	Hi-3	Hi-5	Hi-5
III-e	Hi-15	Hi-17	Hi-48	Hi-41	Hi-1	Hi-18	Hi-1	Hi-5	Neg	Hi-5	Hi-4
III-f	Hi-26	Hi-27		Hi-10	Hi-30		Hi-10	Neg	Hi-5	Hi-5	Unique
III-g	Hi-10	Hi-9	Hi-9			Neg	Neg	Hi-3	Hi-3	Neg	Hi-4
III-h	Hi-10	Hi-9		Hi-32	Hi-27	Hi-30			Hi-3	Hi-18	Neg
III-i	Hi-15	Hi-17	Hi-27	Hi-10	Hi-10	Neg	Hi-5	Hi-5	Neg	Hi-5	Hi-5
III-j		Hi-17	Hi-48	Hi-32	Hi-27	Hi-32	Hi-48	Hi-5	Hi-5	ND	Neg
III-k	Hi-16	Hi-16	Hi-9	Hi-27	Hi-32	Neg	Hi-10	Hi-3	Hi-3	Unique	Neg
III-l	Hi-16	Hi-16	Hi-9	Hi-33	Hi-33	Hi-33	Hi-5	Hi-5	Hi-3	Hi-3	Neg
III-m					Unique	Hi-33	Neg	Hi-3	Hi-3	Neg	Hi-4
III-n		Hi-16	Hi-41	Hi-16	Hi-10		Hi-33	Hi-33		Hi-33	
III-o	Hi-18	Neg	Hi-41	Neg	Hi-1		Neg	Hi-3	Hi-10	Hi-10	Hi-10
III-p	Hi-9	Neg	Hi-41	Hi-32	Hi-32	Hi-33	Hi-28	Neg	Hi-10	Neg	

[a] Neg, no *H. influenzae* detected; Unique, isolate with a unique PFGE profile; ND, PFGE profile not determined; blank cell, not sampled.

(Spain^{23F} ST81), and NT-Pn5 (Norway^{NT} ST344). Clone 19F-Pn3 was by far the most successful of all pneumococcal clones: 68% of the children were colonized by it at least once, and several had it for long periods, resulting in 34.2% of all pneumococcal isolates. In particular, two children (I-f and III-c) carried this clone for more than 42 weeks, and nine of the 13 putative clone reacquisitions were due to it. Clone 23F-Pn1 colonized 11 children only but was isolated on as many as 25 occasions (I/CC of 2.3), suggesting that it had a high capacity for prolonged colonization. By contrast, clone 10A-Pn7 colonized 16 children but was mostly isolated from each child on a single occasion (I/CC of 1.3), suggesting a high degree of transmissibility but low capacity for persistence in the host.

Of interest, three variants of the internationally spread clone Spain^{9V} ST156 were detected: penicillin-resistant strains with serotypes 9V or 14 and penicillin-susceptible variants of serotype 14 (Table 2 and Fig. 1).

The introduction and subsequent dissemination of several clones were detected: clone 6B-Pn6, first detected in week 29, and 6B-Pn9, first detected in week 5, were in circulation

TABLE 4. Pneumococcal clones in circulation during the longitudinal study

Clone	No. of isolates (% of all pneumococcus isolates)	No. of carriers (n = 47)	No. of pneumococcal isolates/no. of children colonized	Range of observed duration of carriage (wks)[a]	Room(s) of isolate(s)	Antibiotype (drug resistance)[b]
19F-Pn 3 (Portugal^{19F} ST177)	88 (34.2%)	33	2.7	1–45	I, II, III	ERY, CLI, TET (14), SXT (5)
23F-Pn1 (Spain^{23F} ST81)	25 (9.7%)	11	2.3	1–31	I, II, III	PEN, CHL, TET, SXT
10A-Pn7	20 (7.8%)	16	1.3	1–13	I, II, III	Susceptible
9V-Pn2 (Spain^{9V} ST156)	8 (3.1%)	4	2.0	1–15	I, III	PEN, SXT
14-Pn2-R (Spain^{9V} ST156)	9 (3.5%)	7	1.3	1–>8*	I, II	PEN, ERY, CLI, TET
14-Pn2-S (Spain^{9V} ST156)	13 (5.1%)	7	1.9	1*	III	ERY, CLI, TET, SXT (8)
6B-Pn9	15 (5.8%)	8	1.9	1–9	I, III	Susceptible
NT-Pn5 (Norway^{NT} ST344)	15 (5.8%)	9	1.7	1–30	I, II, III	PEN, ERY, CLI (8), TET, SXT
23F-Pn14	14 (5.4%)	10	1.4	1–17	I, II	Susceptible
19A-Pn11	14 (5.4%)	9	1.6	1–18	I, II, III	Susceptible
6B-Pn6	11 (4.3%)	5	2.2	≥9*	I, III	Susceptible
11A-Pn8 (ST408)	5 (1.9%)	2	2.5	1–14	I	Susceptible
18F-Pn4	5 (1.9%)	5	1.0	1*	I, II	Susceptible
16F-Pn13 (ST30)	5 (1.9%)	3	1.7	1–22	I, III	Susceptible
6B-Pn12	3 (1.2%)	2	1.5	1–13	I	CHL, ERY, CLI, TET
15A-Pn15 (Sweden^{15A} ST63)	2 (0.8%)	1	2.0	1	I	PEN, ERY, CLI, TET
NT-Pn17	1 (0.4%)	1	1.0	1	II	PEN
NT-Pn18	1 (0.4%)	1	1.0	1	III	PEN, ERY, CLI, TET, SXT
NT-Pn10	1 (0.4%)	1	1.0	1	I	SXT
8-Pn15	1 (0.4%)	1	1.0	1	I	Susceptible
23B-Pn16	1 (0.4%)	1	1.0	1	III	Susceptible

[a] *, study ended; the information obtained is partial.
[b] PEN, penicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim. The number of isolates that were resistant to the antimicrobial agent is indicated in parentheses.

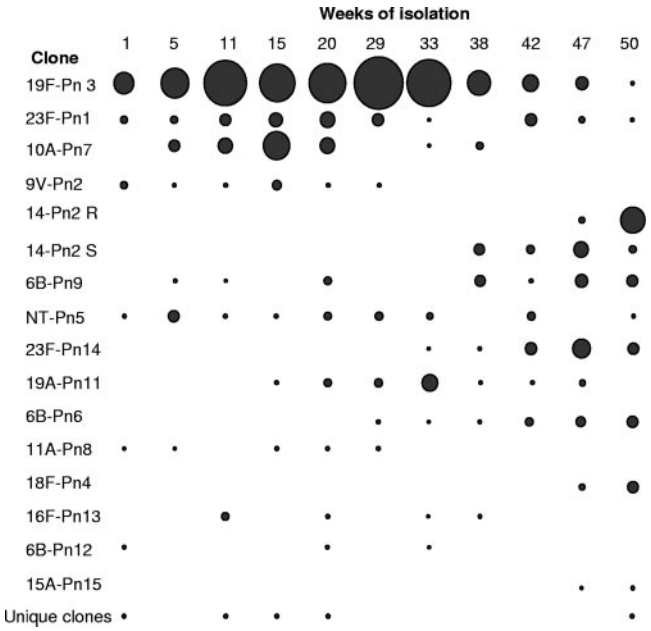

FIG. 1. Temporal patterns of dissemination of pneumococcal clones. The sizes of the circles are proportional to the number of isolates obtained. Unique clones refer to single PFGE patterns.

through the end of the study and colonized five and eight children, respectively. Clone 10A-Pn7 colonized 16 children from the three rooms and circulated between weeks 5 and 38, and clone 19A-Pn11 colonized nine children and circulated between weeks 15 and 47.

***H. influenzae* clones.** PFGE profiles were obtained for 355 of the 360 *H. influenz*ae isolates, and 47 clones were detected (Table 5 and Fig. 2). Three clones (*H. influenzae* clone with PFGE pattern 9 [Hi-9], Hi-10, and Hi-13) were present throughout the study period. Each was isolated in 9 of the 11 sampling periods and colonized children from all three rooms.

Contrary to what was observed for pneumococci, there was no single dominant clone: the most abundant one, Hi-13, accounted for only 10.3% of the isolates. Still, this clone seemed to combine the capacity to persist in the host and to disseminate (I/CC of 2.1) as it was isolated from 18 children (38%). Of 11 putative clone reacquisition events detected, six were due to this clone. Clone Hi-27 (also abundant) also colonized 18 children and demonstrated epidemic behavior. It was first recovered on week 5 from a single child; by week 15 it had been isolated from children in all three rooms and continued to be isolated until week 29. However, despite its successful dissemination, it did not seem to be able to persist in the host over time as only two children were found to carry it consecutively (I/CC of 1.1). Similar behavior was observed for clones Hi-3

TABLE 5. *H. influenzae* clones in circulation during the longitudinal study

Clone	No. of all isolates (% of all *H. influenzae* isolates)	No. of carriers (n = 47)	No. of *H. influenzae* isolates/no. of children colonized	Range of observed duration of carriage (wks)[a]	Room(s) of isolate(s)
Hi-13	37 (10.3%)	18	2.1	1–28	I, II, III
Hi-10	33 (9.2%)	20	1.7	1–28	I, II, III
Hi-7	30 (8.3%)	20	1.5	1–>8	II
Hi-3	28 (7.8%)	22	1.3	1–>5	I, II, III
Hi-5	26 (7.2%)	12	2.2	1–18	II, III
Hi-9	23 (6.4%)	20	1.2	1–10	I, II, III
Hi-27	20 (5.6%)	18	1.1	1–5	II, III
Hi-12	15 (4.2%)	14	1.1	1–≥4	I, II
Hi-48	11 (3.1%)	10	1.1	1	I, II, III
Hi-32	10 (2.8%)	8	1.3	1–14	II, III
Hi-33	9 (2.5%)	5	1.8	1–14	III
Hi-17	8 (2.3%)	6	1.3	1–9	I, II, III
Hi-35	8 (2.3%)	7	1.1	1–4	I, II
Hi-11	8 (2.3%)	6	1.3	1–5	I, II
Hi-16	7 (2.0%)	4	1.8	4–10	I, III
Hi-18	7 (2.0%)	5	1.4	1–9	II, III
Hi-8	6 (1.7%)	6	1.0	ND	I, II
Hi-15	5 (1.4%)	5	1.0	1	I, II, III
Hi-41	5 (1.4%)	5	1.0	1	III
Hi-1	5 (1.4%)	4	1.3	1–13	III
Hi-44	5 (1.4%)	3	1.7	1–14	I, II
Hi-26	4 (1.1%)	4	1.0	1	I, II, III
Hi-19	4 (1.1%)	4	1.0	1	I, II
Hi-28	4 (1.1%)	3	1.3	1–9	I, III
Hi-30	4 (1.1%)	4	1.0	1	III
Hi-14	4 (1.1%)	3	1.3	1–4	I, II
Hi-43	3 (0.8%)	2	1.5	1	I
Hi-4	3 (0.8%)	3	1.0	ND	III
Hi-20	2 (0.6%)	2	1.0	1	I
Hi-31	2 (0.6%)	2	1.0	1	I
Hi-38	2 (0.6%)	2	1.0	1	II
Hi-47	2 (0.6%)	2	1.0	1	I
Unique	15 (4.2%)	1 (each)	1.0	1	I, II, III

[a] ND, not determined since the study ended and information obtained was partial.

and Hi-9. By contrast, clone Hi-5 colonized only 12 children but was isolated on 26 occasions (I/CC of 2.2), suggesting its higher capacity for persistence in the host.

Among the seven most abundant clones, which accounted for 54.8% of the isolates, three (clones Hi-3, Hi-5, and Hi-7) emerged only late in the study and became very abundant in the winter, suggesting that they transmitted well.

The maximum colonization period detected for the same clone of *H. influenzae*, i.e., 28 weeks for clones Hi-10 and Hi-13, was substantially lower than the periods observed for some pneumococci.

Of interest, clone Hi-12 was detected in 33% of children sampled in week 1 but in only two children in week 5 and was not detected afterwards. However, most clones were in circulation for several weeks.

DISCUSSION

To the best of our knowledge, this study represents the largest longitudinal study carried out in a single DCC that has used molecular typing techniques to characterize all pneumococci and *H. influenzae* isolates. Similar colonization studies were conducted by Raymond et al. but in an orphanage (19, 20).

This study gave us the opportunity to thoroughly dissect and follow the dynamics of colonization, persistence, and transmission of these two important bacterial pathogens in the DCC setting. It is the first longitudinal study of this nature to be carried out in Portugal. By targeting a homogeneous group, we were able to focus on identifying pathogens with diverse fitness capacities and hosts with diverse colonization patterns (which were unlikely to be due to age differences) and to understand the dynamics of colonization in the DCC.

We observed high levels of cross-transmission as 15 of the 21 genotypes detected among pneumococci and 32 of the 47 genotypes detected among *H. influenzae* isolates were shared between children. The full extent of this phenomenon becomes even more obvious when absolute numbers of isolates are taken into account: 98% of the pneumococci and 96% of the *H. influenzae* isolates belonged to clones that were shared. In other words, all children were overwhelmingly colonized by clones that were also detected among other attendees.

Although one could postulate that the high degree of sharing results from lack of exposure to a large pool of genetically diverse strains, as observed by Raymond et al. in the orphanage (19, 20), our observations suggest the opposite. In fact, a large number of genetic backgrounds were identified for each pathogen, and novel clones emerged throughout the entire study. In

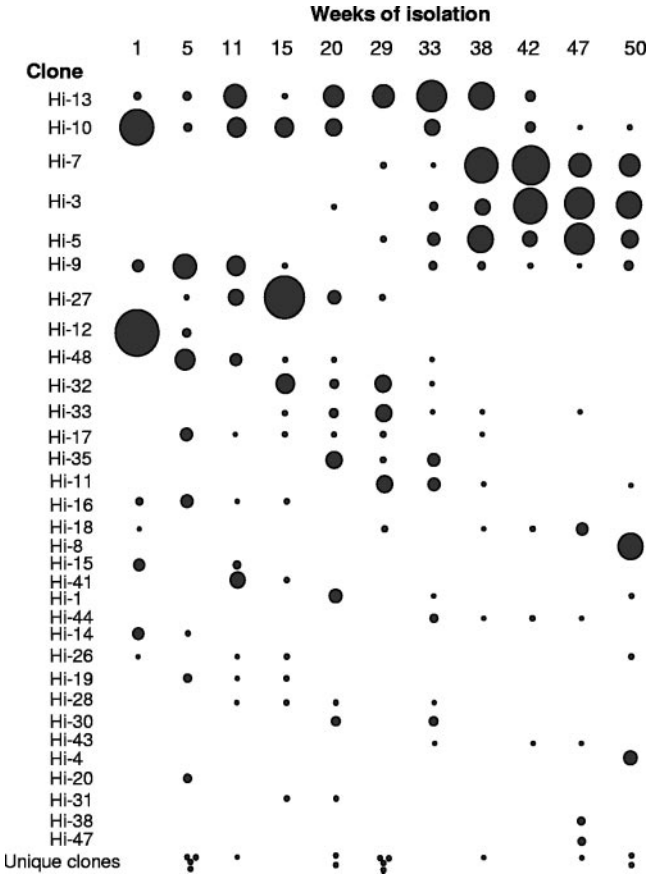

FIG. 2. Temporal patterns of dissemination of *H. influenzae* clones. The sizes of the circles are proportional to the number of isolates obtained. Unique clones refer to single PFGE patterns.

contrast to the children at the orphanage, the DCC attendees were likely to be exposed to other strains from their contacts outside and often introduced these strains into the DCC. Therefore, the most plausible explanation for the high degree of sharing lies in the fact that a significant part of the children's lives was spent in day care (5 days a week, often for 8 h per day or more), providing ample opportunities for cross-transmission.

Focusing on the host revealed that most children appeared to be intermittently colonized, and sequential colonization with as many as six different pneumococcal strains (and five different serotypes) and nine different *H. influenzae* strains was detected. Overall, we estimated that each child was colonized by a mean of 3.6 pneumococcal clones (or 3.4 pneumococcal serotypes) and 7.1 *H. influenzae* clones during the 11 sampling periods spread throughout 1 year. Raymond et al. found that children were colonized by a mean of three pneumococcal and three *H. influenzae* strains per year (19, 20). These observations suggest that the more genetically diverse bacterial pool observed in the DCC contributed to a more dynamic colonization pattern with a greater turnover of strains.

Still, two children carried the same pneumococcal strain for more than 9 months, and another eight children were colonized by the same strain for 5 to 7 months. Such long durations of carriage have also been reported by Gratten et al. in a study in Papua New Guinea (8). Prolonged colonization has been inversely correlated with age but also depends on the host's genetic susceptibility and on the genetic background of the bacteria (7). Of particular interest, 8 of the 10 prolonged colonization episodes were due to the same pneumococcal clone, 19F-Pn3, suggesting that this strain induces a weak immune response and/or out-competes its peers when cocolonization occurs.

Only one child (2%) was never colonized by pneumococci. Raymond et al. found similar "resistance" to pneumococcal colonization in 4% of the children (20). Whether these children have genetic polymorphisms which confer immunity to pneumococcal colonization is unknown although that may well happen as mutations that confer protection against pneumococcal disease have been described previously (11).

Colonization periods of 4 to 6 months with the same *H. influenzae* strain were detected in seven children. Gratten et al. reported long colonization periods of up to 7 months (8). Similar to what was observed for pneumococci, prolonged colonization by *H. influenzae* strains was due to a restricted number of clones: Hi-5, Hi-10, and Hi-13.

Putative reacquisition events were detected for both pneumococci and *H. influenzae* strains in 28% and 19% of the children, respectively. The majority of these events were due to the most abundant clone detected for each species, 19F-Pn3 and Hi-13, both in circulation at the time of redetection. However, the possibility of prolonged undetected colonization should not be discounted and, in fact, has been favored by other investigators (25). As we did not investigate simultaneous colonization by multiple strains, the data should be interpreted cautiously. Other studies have reported that more than 50% of *H. influenzae*-positive samples may contain two or more strains (16, 26), and multiple strains of pneumococci have been observed in 10 to 40% of positive samples (16, 24). Still, true reacquisition of the same pneumococcal serotype but of a different genotype was detected on five occasions.

To focus on the pathogens, we used genotyping to characterize the diversity of the bacterial population in circulation and to detect successful clones that appeared to have increased capacities to persist in the host due to prolonged colonization and/or to rapidly transmit and colonize large numbers of children.

In particular, we identified a pneumococcal clone, 19F-Pn3 (or Portugal^{19F} ST177 [13]), which was extremely successful both in transmission and persistence by all numbers analyzed: it was endemic all year, it was the most abundant clone, it colonized 68% of the children, and it had the highest I/CC (2.7). Whether this success was mainly the result of the serotype expressed or due to the specific genetic background (or a combination of both) is not possible to determine as there were no other serotype 19F clones or capsular variants of this lineage detected.

Interestingly, our study suggested that persistence and transmission can result from independent fitness properties: for example, clone 23F-Pn1 (Spain^{23F} ST81) was prone to induce prolonged colonization, and clones 10A-Pn7 and 19A-Pn11 were successful transmitters. Other clones, although introduced in the day care setting, were never able to transmit to many children.

Among *H. influenzae* isolates, we observed a high degree of

genetic diversity and an apparently greater turnover of strains than for pneumococci. Other investigators have also reported a very high degree of diversity of *H. influenzae* genotypes colonizing DCC children (3, 27). Contrary to what was observed for pneumococci, no single dominant clone was identified, but clones with different fitness properties were observed. Clones Hi-9, Hi-10, and Hi-13 were transmitted throughout the year but had different host persistence properties, with Hi-13 the most successful and Hi-9 the least successful. Clone Hi-13 appeared to be the fittest of all *H. influenzae* clones, and indeed most prolonged colonization and reacquisition events were due to it. Of interest, clone Hi-27 appeared to be very fit in transmission but not in inducing prolonged colonization.

It will be of interest to repeat this type of study in the pneumococcal conjugate vaccine era to determine changes in the patterns of colonization not only by pneumococci but also by *H. influenzae* as interference between these two species has been proposed (18) and replacement disease, in otitis media, has occurred (6).

In conclusion, we observed that novel clones of pneumococci and *H. influenzae* strains were frequently introduced in the DCC and generated a pool of genetically diverse bacteria to which the children were exposed. The DCC attendees represented a pool of hosts in close contact that favored selection of the fittest clones through cross-transmission, with the result that all children were primarily colonized by shared clones. Pneumococcal and *H. influenzae* clones with high propensities for transmission and/or persistence were identified. The further characterization of these clones on a genomic level will be of interest in understanding the bacterial factors leading to successful colonization.

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