Sequential Colonization by *Streptococcus pneumoniae* of Healthy Children Living in an Orphanage

Josette Raymond,1 Isabelle Le Thomas,1 Florence Moulin,2 Anne Commeau,2 Dominique Gendrel,2 and Patrick Berche1

A prospective study of nasopharyngeal colonization by *Streptococcus pneumoniae* in the exceptional conditions of a closed community of abandoned children was done over a 1-year period; 71 children (age <24 months) were studied monthly. *S. pneumoniae* was isolated from 58 (81.7%), and 94.5% of the 111 isolates were resistant to penicillin. The mean rate of carriage was estimated at 57.4%, ranging from 42.8% to 70.4%. Children were sequentially colonized by a mean of 3 different isolates. The mean duration of carriage for a given isolate was ~2.2 months. Serotyping and molecular typing by pulsed-field gel electrophoresis showed that children were colonized by a limited number of clones belonging to only 4 serotypes and 4 pulsortypes. These clones rapidly spread in the community and colonized the children in waves, with a rapid turnover of *S. pneumoniae* isolates, facilitated by close contact between children.

*Streptococcus pneumoniae* is a human pathogen responsible for respiratory infections, including acute otitis media and acute pneumonia, and severe invasive diseases such as meningitis and bacteremia in populations worldwide. It causes high levels of morbidity and mortality despite penicillin treatment. The control of pneumococcal diseases is threatened by the emergence of penicillin resistance, often associated with multiple antibiotic resistance. The prevalence of penicillin resistance among pneumococci, both high-level (MIC, >1 μg/mL) and intermediate (MIC, 0.1–1 μg/mL), is rapidly increasing worldwide [1]. There is evidence that this reflects the spread of a restricted number of multiresistant pneumococcal clones [2, 3]. Pneumococcal infection is usually preceded by colonization of the human nasopharynx [4], indicating that nasopharyngeal colonization is an important risk factor for developing disease. For instance, young children frequently colonized by pneumococci develop acute otitis media more frequently than do children colonized less frequently or not at all [5, 6]. *S. pneumoniae* is carried nasopharyngeally by 20%–40% of healthy children, and the rate of nasopharyngeal carriage is much lower in adults [4]. Most children are colonized by *S. pneumoniae* during the first 2 years of life, depending on epidemiologic and socioeconomic factors [7]. In the United States, the first pneumococcal isolates were found to be acquired at 6 months of age [4], whereas in Papua New Guinea colonization occurs as early as 1 month of age [8]. Because *S. pneumoniae* is transmitted mainly by contacts between individuals, the close contact between young children in day care centers increases the rate of nasopharyngeal carriage of *S. pneumoniae* [9]. However, little is known about the natural history of *S. pneumoniae* colonization and the turnover of isolates among young children over a long period of time.

In this work, nasopharyngeal colonization by *S. pneumoniae* was followed over a 1-year period in children living in an orphanage. The initial aim of this prospective study was to evaluate the incidence of penicillin-resistant *S. pneumoniae* to select an antimicrobial treatment that is likely to be effective in these children. This orphanage, in which the children live in a closed community, represents an extreme version of the situation in a day care center. This prospective study enabled us to study the kinetics of acquisition of *S. pneumoniae* isolates in this community and to determine the clonal diversity and transmission of isolates in children.

**Children and Methods**

*Children*. We studied nasopharyngeal colonization by *S. pneumoniae* in children living in an orphanage at Saint-Vincent de Paul Hospital, which houses healthy orphans and children abandoned in the urban area of Paris. Children were admitted to the health-care unit, comprising 50 beds in a single room divided into eight cubicles and covering an area of 130 m². Children lived, played, slept, and received care nights and days in the same room.

*Sampling and cultures*. Nasopharyngeal samples were obtained by use of a flexible calcium alginate swab, which was introduced into the nostrils until resistance was encountered. Swabs were rapidly used to inoculate trypticase soy agar containing 5% sheep...
mosomal DNA of susceptibility to penicillin, and those with MICs $>0.125$ were further tested by the E-test with penicillin and cefotaxime. For cefotaxime, strains with MICs $>0.5$ and $<2.0$ mg/mL were considered to be of intermediate susceptibility. One colony from each primary culture was selected for further investigation. Susceptibility to cotrimoxazole, tetracycline, erythromycin, lincomycin, pristinamycin, rifampicin, chloramphenicol, vancomycin, and teicoplanin was determined by the disk-diffusion method of Bauer and Kirby (Mueller-Hinton agar). Serotyping was done on bacterial suspensions derived from single colonies of *S. pneumoniae*. Serotyping was done at the National Streptococcus Reference Laboratory, Creteil (Dr. P. Geslin), by means of the Quellung reaction with specific antisera provided by the Statens Serum Institut (Copenhagen).

We used strain R6 as well as 3 endemic strains of *S. pneumoniae* (1485, 1674, and 1678) as controls, as described elsewhere [11].

**Pulsed-field gel electrophoresis (PFGE).** Molecular typing was done on bacterial suspensions derived from single colonies of *S. pneumoniae*. Bacterial suspensions were prepared in Tris-EDTA (TE; $10^{-2}$ M Tris base and $10^{-3}$ M EDTA, pH 7.5; Sigma, Saint Quentin Fallavier, France), supplemented with 7% sucrose, and adjusted to an optical density of 1.2 at 650 nm. This suspension was mixed with an equal volume of 2% low-melting-point agarose (Sigma). The molten mixture was poured into 4 perspex molds (100 μL). The agarose was allowed to solidify, and the cells embedded in it were incubated in lysozyme solution (20 mg/mL) for 1 h at 37°C. Complete lysis was then achieved by incubation overnight at 50°C in 0.5 M EDTA, pH 8, 50 μg/mL proteinase K (Sigma), and 1% (wt/vol) SDS. Proteinase K was inhibited by adding 1 mM phenylmethylsulfonyl fluoride and incubating at room temperature for 1 h. Plugs were then washed with 1× TE buffer, next with 0.5× TE, and finally with distilled water. For digestion of DNA, the agarose plugs were equilibrated by incubation with 1× restriction buffer for 30 min. The restriction enzyme (15 U; New England Biolabs, Beverly, MA) was then added. DNA was digested overnight with *Smal*, and the fragments were separated on a 1% agarose gel by PFGE (CHEF mapper DR II; Bio-Rad Laboratories, Richmond, CA) for 24 h at 14°C at 6 V/cm with pulse times of 8–35 s and an angle of 120°. Gels were stained with ethidium bromide and viewed under UV light.

The coefficient of similarity, (number of shared bands $× 2$ and total number of bands in the two samples, and the Dice index were determined for each isolate, by use of the computer program Bio-Profil (Vilber Lourmat, Marne la Vallée, France). A dendrogram was produced by the unweighted pair-group method. Strains with a coefficient of similarity $>80$% were considered to belong to the same lineage.

**Statistical analysis.** All duplicate pathogens were counted as 1 isolate per patient. The Epi Info statistics program (Centers for Disease Control and Prevention, Atlanta), Student’s $t$ test, and the $χ^2$ test were used for statistical comparisons.

**Results**

*Incidence of nasopharyngeal* *S. pneumoniae* *colonization in healthy children.* A total of 71 children living in an orphanage, to which they were admitted from birth to the age of 24 months, were included in this study from January to December 1996. Nasopharyngeal specimens were collected monthly for 53 children $>6$ months of age and every 2 weeks for 18 children $<6$ months of age. New admissions to the orphanage were systematically enrolled. *S. pneumoniae* was isolated from at least 1 nasopharyngeal culture for 58 (81.7%) of the 71 children and from a total of 167 (57.8%) of 289 samples. The mean rate of carriage was estimated at 57.4%, ranging from 42.8% to 70.4%.
S. pneumoniae did children without pneumoniae isolates had a longer stay (mean, 7.3 months) than pulsotypes corresponding to 167 isolates of S. pneumoniae belonging to 4 main serotypes, showing genetic relationship between 17 (50 mg/kg/day; 15.4%), amoxicillin (50 mg/kg/day; 10.2%), co-amoxicillin±clavulanic acid (100 mg/kg/day; 69.2%), josamycin (500 mg/kg/day; 15.4%), and penicillin (100,000 IU/kg/day; 2.6%). S. pneumoniae was less frequently isolated from children who had been treated with antibiotics 15 days before (16/39 samples; 41%), than from untreated children (151/250 samples; 60.4%; \( P = .02 \)). This suggests that prior antibiotic treatment (<15 days previously) may decrease the carriage of penicillin-resistant isolates. The penicillin MIC of the isolates was not affected by prior treatment (\( P > .15 \)).

**Penicillin resistance of S. pneumoniae isolates.** Fifty-six of the 167 S. pneumoniae isolates were repeatedly cultured from the same children during the study. Therefore, only 111 isolates were analyzed further. Ninety-five (94.5%) of these 111 isolates displayed a decreased susceptibility to penicillin, including 55 (58.5%) with intermediate penicillin resistance and 40 (36%) fully penicillin-resistant. Only 6 children were colonized with penicillin-susceptible isolates. Forty-eight (43.2%) isolates were susceptible to cefotaxime. Decreased susceptibility to cefotaxime was observed in 63 (56.8%) of 111 isolates, and only 1 isolate was resistant to this antibiotic. Most S. pneumoniae isolates with decreased susceptibility to penicillin were resistant to macrolides (97%) or cotrimoxazole (88%), and a large proportion were resistant to chloramphenicol (53%) and to tetracycline (65%). In contrast, most penicillin-susceptible isolates were susceptible to macrolides, tetracyclines, and cotrimoxazole. Most S. pneumoniae isolates belonged to 4 capsular serotypes: serotype 23F (27%), serotype 19F (22%), serotype 14 (22%), and serotype 6B (20%). Only 1 isolate of serotype 9 was found. Three of the 6 children colonized with penicillin-susceptible isolates were present in the orphanage for only 1 week and 1 child for 3 months. The other 2 were present for 8 and 9 months, respectively, but returned to their families a few days before the isolation of S. pneumoniae. This suggests that penicillin-susceptible isolates, except in 1 case, originated from the surrounding community and failed to diffuse in the orphanage. No seasonal increase of pneumococcal carriage was observed during this study.

**Clonal nature of S. pneumoniae isolates.** Molecular typing was done by PFGE with 111 S. pneumoniae isolates obtained from nasopharyngeal cultures. Smal-digested chromosomal DNA generated ~13 fragments of 49–384 kb, defining 12 pulsotypes. Only 1 lineage was identified in penicillin-resistant isolates from the 6B, 14F, and 23F serotypes and 2 for the 19F serotype, suggesting widespread diffusion of penicillin-resistant clones in children. For example (figure 1), the penicillin-resistant isolates from serotype 23F are identical or closely related to the predominant clone 23F previously described in France [11]. All isolates with very similar PFGE patterns belonged to the same serotype. In contrast, clonal diversity was observed in penicillin-susceptible isolates, which were distributed in 6 pulsotypes belonging to various serotypes, as illustrated in the dendrogram in figure 2. We also checked for multiple colonization in 3 children by picking 10 well-separated colonies from primary cultures of nasopharyngeal specimens. All colonies
Sequential nasopharyngeal colonization by *S. pneumoniae* isolates in healthy children. The mean age at first acquisition of *S. pneumoniae* among children arriving at the orphanage was 3 months (range, 2–5). The mean duration of carriage of a given isolate was 2.2 months (range, 2 weeks–6 months). We found that children were sequentially colonized by an average of 3 different isolates (range, 1–4) during the course of 1 year. The mean duration of carriage was 1.9 months (range, 3 weeks–5 months), 2.2 months (range, 3 weeks–4 months), 2.3 months (range, 2 weeks–4.5 months), and 2.4 months (range, 3 weeks–6 months) for serotypes 19F, 6B, 23F, and 14, respectively (not significant), and therefore did not depend on serotype. The mean time to colonization with a new isolate was 3.5 months. Of 40 children aged 3 months–2 years who were present during the entire study, 8 reacquired an isolate of the same serotype. In 5 cases, the serotype and PFGE pulsotype were identical to those of the initial isolate. The time between the first isolation and reacquisition was 2–5 months. In the other 3 cases, the serotypes were identical to those of the initial isolate, but the PFGE pulsotype was not, indicating genetically unrelated isolates from 3 children, as illustrated in figure 3.

During the study, we observed that a given clone rapidly spread in the community and was then eliminated. Thus, 4 main clones belonging to serotypes 6B, 14, 19F, and 23F spread in children (figure 4). Isolates belonging to serotype 23F and pulsotype I were cultured for the first time in January 1996 and colonized 76% of the children. The incidence of this clone then decreased in the population, and the clone completely disappeared in July 1996. The serotype 14 clone was isolated from March to June 1996, with an early peak of incidence in March followed by rapid dissemination in the community. Clone 19F diffused more slowly than did serotypes 14 and 6B. Serotype 19F pulsotype I was first isolated in March 1996, and its incidence peaked during the summer of 1996. Serotype 19F pulsotype II appeared in January 1996, disappeared for 8 months, and then reappeared, colonizing only 1 child. The main clone of serotype 6B (pulsotype I) diffused rapidly in 1 month (July 1996) and then persisted (40% of the isolates) until December 1996. The other 7 isolates (1 penicillin-resistant isolate, serotype 9, and 6 penicillin-susceptible isolates) were cultured only once.

Discussion

A prospective study was performed over a 1-year period in the exceptional conditions of a closed community of orphans and abandoned children. This enabled us to study the kinetics of nasopharyngeal colonization by *S. pneumoniae* and the transmission of this pathogen among young children living in the same room for a prolonged period with very few outside contacts. As expected, the rate of *S. pneumoniae* carriage (~58%) was similar to those reported in day care centers [12–16] but was higher than those reported for children living in the community (~25%–40%) [4, 17]. Several risk factors have been associated with *S. pneumoniae* carriage, including day care center attendance [4, 14, 18, 19], the size of the day care center [20], living in close contact as siblings [21], and socioeconomic factors, including a smaller living area [9, 22]. The rate of *S. pneumoniae* carriage (10.8%) reported for Chinese children living in
that children were sequentially colonized by various clones, which rapidly spread in a wavelike manner and were replaced by new clones. These waves were not correlated with antibiotic exposures. We cannot exclude the possibility that children were colonized with several isolates, because we investigated the clonal nature of the isolates from only 3 children. However, coinfection occurs in only a few cases (0.28%–16%) [25, 26]. The mean duration of carriage for a given isolate was estimated to be 2.2 months in our study. It has been reported that the duration of carriage depends on the child’s age [4] and the serotype [21, 27], and longer durations of carriage ≤17 months have been reported, although they were not checked with molecular markers. We observed that children were reinfected in 20% of cases with an isolate of the same serotype. A reinfection rate of 30% has been reported elsewhere [4]. PFGE showed that the second isolate had the same genotype in only 5 cases and that, in 3 cases, a genetically different clone with the same serotype was isolated, suggesting a transfer of the gene cluster encoding capsular antigens, as described elsewhere [28]. This result suggests that children were poorly immunized against capsular antigens after the primary colonization. In this study, we determined the age at first colonization for 7 of 71 children; it was estimated to be ~3 months. Twelve percent of children attending a well-baby clinic in Sweden were colonized at 2 months of age [9], whereas, in Papua New Guinea, most children were found to be colonized with S. pneumoniae by 1 month of age [8]. Interestingly, all serotypes observed in our study are included in the new pneumococcal conjugate vaccines in development or in trials, and if conjugate vaccines can decrease overall carriage rates in children, decreased transmission would be observed [29].

In conclusion, this study, performed in the unique epidemiologic conditions of a closed community, provides new insight into the natural course of nasopharyngeal colonization by a major human pathogen.

Acknowledgments

We are grateful to E. Abachin and G. Quesne for advice on pulsed-field gel electrophoresis and to A. Ferroni (Hôpital Necker-Enfants Malades) for providing reference strains.

References

5. Faden H, Duffy L, Wasielewski R, et al. Relationship between nasopharyn-