JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1998, p. 2248–2253 Vol. 36, No. 8
0095-1137/98/$04.00+0
Copyright © 1998, American Society for Microbiology. All Rights Reserved.

Molecular Characterization of Pneumococcal Nasopharynx Isolates Collected from Children during Their First 2 Years of Life

MARCEL SLUIJTER,[1] HOWARD FADEN,[2] RONALD DE GROOT,[1] NICOLE LEMMENS,[3] WIL H. F. GOESSENS,[3] ALEX VAN BELKUM,[3] AND PETER W. M. HERMANS[1]*

Department of Pediatrics[1] and Department of Medical Microbiology & Infectious Diseases,[3] Erasmus Medical Center Rotterdam, Erasmus University Rotterdam, 3000 DR Rotterdam, The Netherlands, and Department of Pediatrics, Division of Infectious Diseases, School of Medicine and Biomedical Sciences, Buffalo, New York[2]

Received 26 January 1998/Returned for modification 9 April 1998/Accepted 5 May 1998

Pneumococcal colonization was studied in 19 children monitored from birth through the age of 2 years. For this purpose, pneumococcal isolates were characterized by capsular typing, restriction fragment end labeling (RFEL), and penicillin-binding protein (PBP) genotyping. Fifty-eight isolates were collected and were found to belong to 10 capsular types, 31 RFEL types, and 7 PBP genotypes. Thirty-nine percent of the isolates had reduced susceptibility to penicillin. All seven highly resistant strains (MICs, >1 μg/ml) were identical to the pandemic clone 23F. Children were culture positive between one and eight times at 13 scheduled visits. Although the infants were frequently recolonized with different strains, colonization with one particular strain often persisted for several months. Isolation of a previously detected capsular type was common, and the chromosomal homogeneity tended to be high when it occurred. Horizontal transfer of capsular genes between strains of different RFEL types was demonstrated in one child. The ecological advantage of transfer of capsular genes is unclear unless survival of the organism on a mucosal surface may be linked to immunoprotective pressure against particular capsular types.

Streptococcus pneumoniae (pneumococcus) is a major cause of invasive disease such as meningitis, bacteremia, and pneumonia, as well as noninvasive disease like acute otitis media and sinusitis (3). In young children, the pneumococcus is often part of the normal nasopharyngeal flora. Especially during the first 2 years of life, children are colonized with novel strains of pneumococci. Children colonized with *S. pneumoniae* in the nasopharynx develop acute otitis media more often than children who are not colonized (9, 14, 25).

The increasing rate of antibiotic resistance in *S. pneumoniae* complicates the elimination of pneumococcal strains from the middle ear and strongly supports the application of new vaccine strategies. Conjugate capsular vaccines contain a limited number of capsular serotypes, linked to a carrier protein (2, 5). Although the results of early trials with these vaccines look promising, care should be taken since several investigators have observed horizontal transfer of capsular genes (1, 11, 12). Horizontal transfer of capsular genes may interfere with vaccination programs in the long run if (antibiotic-resistant) strains with a vaccine-type capsule switch to nonvaccine capsular types.

The present report describes the longitudinal changes in pneumococcal colonization of the nasopharynx in a cohort of children monitored from birth through 2 years of age. Pneumococcal isolates were characterized by capsular typing, restriction fragment end labeling (RFEL), BOX PCR typing, antibiotic susceptibility testing, and penicillin-binding protein (PBP) genotyping.

MATERIALS AND METHODS

Nineteen children were enrolled in their first month of life without regard to sex, race, or social status. Informed consent was obtained from all parents at the time of entry into the study. Children were excluded if they were born prematurely or had significant congenital anomalies, especially those involving the face and head.

Children were examined at monthly intervals for the first 6 months and at 8, 10, 12, 15, 18, 21, and 24 months. At the initial examination and at each subsequent examination, the nutritional history was obtained. At the end of the period of observation, information concerning siblings in the household and attendance at day care was registered. Ear examinations by pneumatic otoscopy were performed at each scheduled visit to determine the presence of acute otitis media. Tympanometry was performed for all subjects older than 6 months. Interim histories were also obtained to determine episodes of acute otitis media that may have occurred between scheduled visits and to determine the antibiotics which had been used.

Nasopharyngeal swab specimens for culture were obtained at each scheduled visit, as described before (9). *S. pneumoniae* was identified by colonial morphology, Gram staining, bile solubility, and optochin susceptibility (18). From each primary culture, one colony was picked for further analysis. Serotyping was performed on the basis of capsular swelling by using commercial antisera (8).

Susceptibilities to penicillin, trimethoprim-sulfamethoxazole, doxycycline, erythromycin, rifampin, vancomycin, and ciprofloxacin were tested by the agar dilution method as described before (12). Breakpoints for intermediate or high-level resistance were used according to the guidelines of the National Committee for Clinical Laboratory Standards for susceptibility testing (19).

RFEL was performed as described previously (13, 24). In short, cetyltrimethylammonium bromide-purified pneumococcal DNA was digested with *Eco*RI and was radioactively labeled with [α-^{32}P]dATP at 72°C with DNA polymerase (Goldstar; Eurogentec, Seraing, Belgium). The DNA restriction fragments were denatured and separated electrophoretically on a 6% polyacrylamide gel containing 8 M urea for 4 h at 100 W. The gel was transferred onto filter paper (Whatman 3MM), vacuum dried, and exposed to ECL hyperfilm (Amersham) at room temperature for various lengths of time. RFEL patterns were analyzed with Gelcompar software, version 4 (Applied Maths, Kortrijk, Belgium). DNA fragments in the molecular size range of 160 to 400 bp were scanned with an Image Master DTS scanner (Pharmacia Biotech, Uppsala, Sweden). The patterns were normalized with the pneumococcus-specific bands present in the RFEL banding patterns of all strains. Comparison of the patterns was performed by the unweighted pair group method with arithmetic averages and by using the Jaccard similarity coefficient applied to peaks. The software package was used according to the instructions of the manufacturer. A tolerance of 1.5% in band positions was applied during comparison of the patterns. DNA types were arbitrarily defined, being identical when RFEL patterns showed homologies of greater than 95%.

BOX PCR genotyping was carried out as described before (23). Briefly, 50 ng of pneumococcal DNA was amplified by PCR (40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 74°C) with primer BOX-A, which is based on the primary structure of the BOX repeat motif (16). The amplified products were separated on a 1.5% agarose gel. The gels were stained with ethidium bromide, after which

* Corresponding author. Mailing address: Laboratory of Pediatrics/Room Ee 1500, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands. Phone: 31-10-4088224. Fax: 31-10-4365053. E-mail: hermans@kgk.fgg.eur.nl.

TABLE 1. Demographic characteristics of study population according to increasing number of nasopharyngeal swab cultures positive for *S. pneumoniae*[a]

Subject	Sex	Nutrition	Siblings	Day care	AOM (no. of episodes)	Colonization (no. of positive samples)[b]
1.R	F	F	Y	N	1	1
2.B	F	B	Y	N	0	1
3.T	M	B	N	Y	0	1
4.B	M	B	Y	Y	3	1
5.G	M	B	Y	N	4	1
6.L	F	B	Y	N	0	2
7.S	M	F	Y	Y	3	2
8.S	M	B	Y	N	5	2
9.B	F	B	N	N	0	3
10.M	F	B	Y	N	2	3
11.R	F	F	Y	Y	3	3
12.K	F	F	Y	Y	4	3
13.K	F	F	Y	N	6	4
14.S	M	F	Y	Y	6	4
15.K	F	B	Y	N	2	5
16.P	F	F	Y	Y	3	5
17.W	M	F	N	N	4	5
18.B	F	F	Y	N	5	5
19.P	F	F	N	Y	5	8

[a] All individuals were Caucasian. Abbreviations: F, female; M, male; F, formula; B, breast; Y, yes; N, no; AOM, acute otitis media.

[b] The results represent the number of samples positive by culture (a total of 13 samples were obtained from each child).

the banding patterns were evaluated visually. Strains with BOX PCR patterns showing one band difference were defined as having nonidentical genotypes.

PBP genes 1A, 2B, and 2X were characterized by restriction fragment length polymorphism analysis. The genes were amplified by PCR as described previously (4, 6, 17). Briefly, 10 ng of pneumococcal DNA was used to amplify each of the PBP genes. The amplification products (5 μl) were digested with the restriction endonuclease *Hin*fI and were separated electrophoretically in 2.5% agarose gels. The gels were stained with ethidium bromide and were photographed with a Polaroid MP4 Landcamera and Polaroid 667 film. The different PBP genotypes are represented by a three-digit code (i.e., 7-5-1), which refers to the restriction fragment length polymorphism patterns of PBP genes 1a (7), 2b (5), and 2x (1), respectively.

RESULTS

As part of a large cohort study, children were monitored from birth to determine the frequency of acute otitis media during their first 2 years of life. Nasopharyngeal swab specimens for culture were collected at regularly scheduled visits at 1 to 6, 8, 10, 12, 15, 18, 21, and 24 months. Nineteen children who completed all 13 scheduled visits and from whom *S. pneumoniae* was grown from nasopharyngeal swab specimens on one or more occasions were included in this study.

Table 1 displays the demographic characteristics of the children. All children were Caucasian. There were 12 females and 7 males. Ten of the children were formula fed and 9 were breast-fed. The vast majority ($n = 15$) had siblings. Eleven were in day care by 2 years of age, whereas eight were not. The number of episodes of acute otitis media varied from 0 to 6, and the median number was 3. The number of times that children were culture positive with *S. pneumoniae* varied from one to eight, and the median number was three.

All 58 pneumococcal isolates were subjected to capsular typing, RFEL typing, BOX PCR typing, antibiotic susceptibility testing, and PBP genotyping. The phenotypic and genotypic characteristics of the pneumococcal strains carried by each individual are summarized in Table 2.

Nine capsular groups, groups 1, 6, 11, 14, 15, 16, 18, 19, and 23, were identified. Type 6 was most common. Strains of this type were observed 20 times and were isolated from 11 children. One strain recognized by commercial pool G could not be typed in further detail.

RFEL typing of 56 pneumococcal strains displayed 31 distinct RFEL types. The genetic relatedness of the strains on the basis of RFEL analysis is depicted in Fig. 1. Twenty RFEL types were unique because they occurred in the population only once. Eleven types occurred two or more times (RFEL clusters). RFEL type 15 was the most dominant RFEL type because it was observed seven times among four children (Table 2). Strains of RFEL type 23 were isolated three times and and were isolated from three children. RFEL type 1 was isolated from two children. From child 3.T, a strain of this type was isolated once and from child 19.P a strain of this type was isolated three times. The three RFEL type 1 isolates from child 19.P (isolates 19.P-1, 19.P-2, and 19.P-6) had identical BOX PCR types (data not shown), as well as identical PBP types (Table 2). However, strain 19.P-1 and 19.P-2 belonged to capsular type 6, whereas strain 19.P-6 belonged to serotype 23. The RFEL type 15 strains 19.P-4 and 19.P-5, which were both isolated prior to the isolation of strain 19.P-6, also had capsular type 23. These data suggest that the genes encoding capsular type 23 were transferred within child 19.P from the RFEL type 15 strain to the RFEL type 1 strain (Table 2). Strains of RFEL type 56 were also isolated from two children. Three of these strains isolated from child 13.K were capsular type 23 and had identical PBP types (7-5-1). The RFEL type 56 strain isolated from child 15.K also had this PBP type. However, this strain had a different capsular type (serogroup 6).

In this study, pneumococcal strains were cultured from the nasopharyngeal swab specimens from 13 children on two or more occasions. For 8 of 13 children, a single strain with the same RFEL type was repeatedly isolated over time, indicating persistent colonization. For the isolates from six children, the isolates were confirmed to be identical by BOX PCR typing. For the isolates from two individuals, the BOX PCR types of strains with identical RFEL types were different, albeit strongly related (types 9B-1 and 9B-2 and types 17W-1 and 17W-2; data not shown).

Antibiotic susceptibility tests showed that for 33 of 54 (61%) strains tested penicillin MICs were <0.1 μg/ml. Twenty-one strains displayed reduced susceptibility to penicillin: 7 strains were highly resistant (MICs, >1 μg/ml) and 14 strains were intermediately resistant (0.1 μg/ml ≤ MICs ≤ 1 μg/ml). Eighteen strains (33%) were resistant to trimethoprim-sulfamethoxazole (MICs, ≥4 μg/ml), whereas eight strains (15%) were resistant to doxycycline (MICs, ≥4 μg/ml). Resistance to two or more antibiotics was observed among 17 strains (31%). Within the group of penicillin-resistant isolates ($n = 21$), 76 and 38% of the strains were coresistant to trimethoprim-sulfamethoxazole and doxycycline, respectively. All pneumococcal isolates were susceptible to rifampin, vancomycin, and ciprofloxacin.

PBP genotyping of 55 pneumococcal strains revealed 10 distinct PBP genotypes. PBP types 2-2-3 ($n = 15$), 2-2-2 ($n = 11$), and 2-2-71 ($n = 8$) occurred most frequently. Except for strain 15.K-2 (penicillin MIC, 0.5 μg/ml), these genotypes exclusively represent the penicillin-susceptible phenotype. Penicillin resistance occurred among isolates with seven distinct PBP types. The seven highly penicillin-resistant isolates of RFEL type 15 invariably had the most frequent type, type 1-1-1. The other six penicillin-resistant PBP types were linked to strains with an intermediate-resistant phenotype. Comparison of the RFEL patterns with those of a collection of 205 international penicillin-resistant pneumococcal strains from 15 countries and with 84 distinct RFEL types (11) showed greater than 95%

TABLE 2. Phenotypic and genotypic characterization of pneumococcal strains

Subject and isolate[a]	Sero-group	Type by RFEL analysis	Type by PBP genotyping	MIC of penicillin (μg/ml)	Resistance pattern[b]
1.R, 1	6	23	2-2-71	<0.1	Susc
2.B, 1	6	22	2-2-2	<0.1	Susc
3.T, 1	6	1	2-2-2	<0.1	Susc
4.B, 1	6	23	2-2-71	<0.1	Susc
5.G, 1	23	15[c]	1-1-1	2.0	PCD
6.L, 1	19	19	2-2-71	<0.1	Susc
7.S					
1	6	7	2-2-2	<0.1	Susc
2	6	5	2-2-2	<0.1	Susc
8.S					
1	16	10	2-2-3	U[d]	U
2	19	85[c]	2-2-46	0.125	PC
9.B					
1	18	30	2-2-71	<0.1	Susc
2	18	30	2-2-71	<0.1	Susc
3	18	U	2-2-71	<0.1	Susc
10.M					
1	6	25	2-2-45	<0.1	C
2	6	U	U	U	Susc
11.R					
1	6	23	2-2-71	<0.1	Susc
2	6	24	U	<0.1	Susc
3	G[e]	9	2-2-71	U	U
12.K					
1	11	13	2-2-3	<0.1	Susc
2	23	14	2-2-2	<0.1	Susc
3	23	14	2-2-2	<0.1	Susc
4	23	14	2-2-2	<0.1	Susc
13.K					
1	23	56[c]	7-5-1	1.0	PC
2	6	91[c]	2-2-3	<0.1	Susc
3	23	56[c]	7-5-1	0.5	PC
4	23	56[c]	7-5-1	0.5	PC
14.S					
1	14	2	U	<0.1	Susc
2	19	90[c]	2-2-12	0.125	P
3	23	15[c]	1-1-1	2.0	PCD
4	23	15[c]	1-1-1	2.0	PCD
15.K					
1	6	4	2-2-3	<0.1	Susc
2	6	91[c]	2-2-3	0.5	PD
3	6	56[c]	7-5-1	0.5	PC
4	23	15[c]	1-1-1	2.0	PCD
5	23	15[c]	1-1-1	2.0	PCD
16.P					
1	1	31	2-2-3	<0.1	Susc
2	1	31	2-2-3	<0.1	Susc
3	1	31	2-2-3	<0.1	Susc
4	15	8	2-2-3	<0.1	Susc
5	11	13	2-2-3	<0.1	Susc
17.W					
1	19	27	2-2-3	<0.1	Susc
2	19	27	2-2-3	<0.1	Susc
3	6	89[c]	1-16-47	0.5	PC
4	6	89[c]	1-16-47	0.5	PC
5	6	89[c]	1-16-47	0.5	PC
18.B					
1	14	87[c]	2-15-44	0.125	P
2	14	87[c]	2-15-44	0.125	P
3	14	88[c]	2-15-44	0.125	P
4	19	86[c]	4-2-46	0.25	PC
5	19	32	2-2-2	U	U
19.P					
1	6	1	2-2-2	<0.1	Susc
2	6	1	2-2-2	<0.1	Susc
3	14	20	2-2-3	<0.1	Susc
4	23	15[c]	1-1-1	2.0	PCD
5	23	15[c]	1-1-1	2.0	PCD
6	23	1	2-2-2	<0.1	Susc
7	6	17	2-2-3	<0.1	Susc
8	19	22	2-2-3	<0.1	C

[a] Isolates are ordered in chronological order.
[b] P, penicillin; C, trimethoprim-sulfamethoxazole (co-trimoxazole); D, doxycycline; susc, susceptible to all antibiotics tested.
[c] RFEL numbering of the penicillin-resistant strains is in concordance with the numbering used by Hermans et al. (11).
[d] U, unknown.
[e] Commercial pool G.

homology between the seven RFEL type 15 strains and the RFEL patterns harbored by the multiple drug-resistant pandemic clone 23F. The PBP type of this international clone 23F and the RFEL type 15 isolates from the children were invariably of type 1-1-1. The strains of RFEL type 56 displayed 100% homology with a strain isolated in Spain. However, the capsular type and PBP type of the Spanish isolate did not match those of the strains isolated from the children. The RFEL types of all other penicillin-resistant colonization isolates described in this study displayed less than 95% homology with any of the 84 RFEL types present in the international collection (data not shown).

DISCUSSION

Children acquire *S. pneumoniae* as part of their normal flora early in life. In the first year of life, the prevalence rate is approximately 20%. Cumulative acquisition rates exceed 50% by 1 year of age (9). In many studies, nasopharyngeal colonization rates in young children are described, but children are rarely monitored for a prolonged period of time. As a part of a large cohort study, the present study monitored the nasopharyngeal carriage of *S. pneumoniae* in 19 children from birth through age 2 years. In this period, nasopharyngeal swab specimens for culture were taken 13 times. An earlier study demonstrated a positive correlation between the frequency of colonization and the number of acute otitis media events (9). In addition, this study further demonstrated that the number of acute otitis media events was significantly higher in the group of formula-fed children (7).

Gray and coworkers (10) demonstrated the dynamic state of nasopharyngeal colonization in children by using capsular typing to monitor longitudinal changes in young children. However, genetically different pneumococcal strains can have the same capsular type (13), suggesting that children carrying a particular serotype for a longer period may be colonized or recolonized with different strains. This feature was clearly observed in the present study. Strains of different RFEL types but identical serotypes were isolated from 5 of 19 individuals. Therefore, genetic typing methods should be used to supplement capsular typing in epidemiological studies.

The pneumococcal isolates in this study were characterized by four different methods of typing. RFEL typing was used to discriminate between genotypes. Although BOX PCR typing has a higher discriminatory power (23), RFEL typing was preferred as the major typing method because the data obtained by that method are easier to analyze by computer analysis (13).

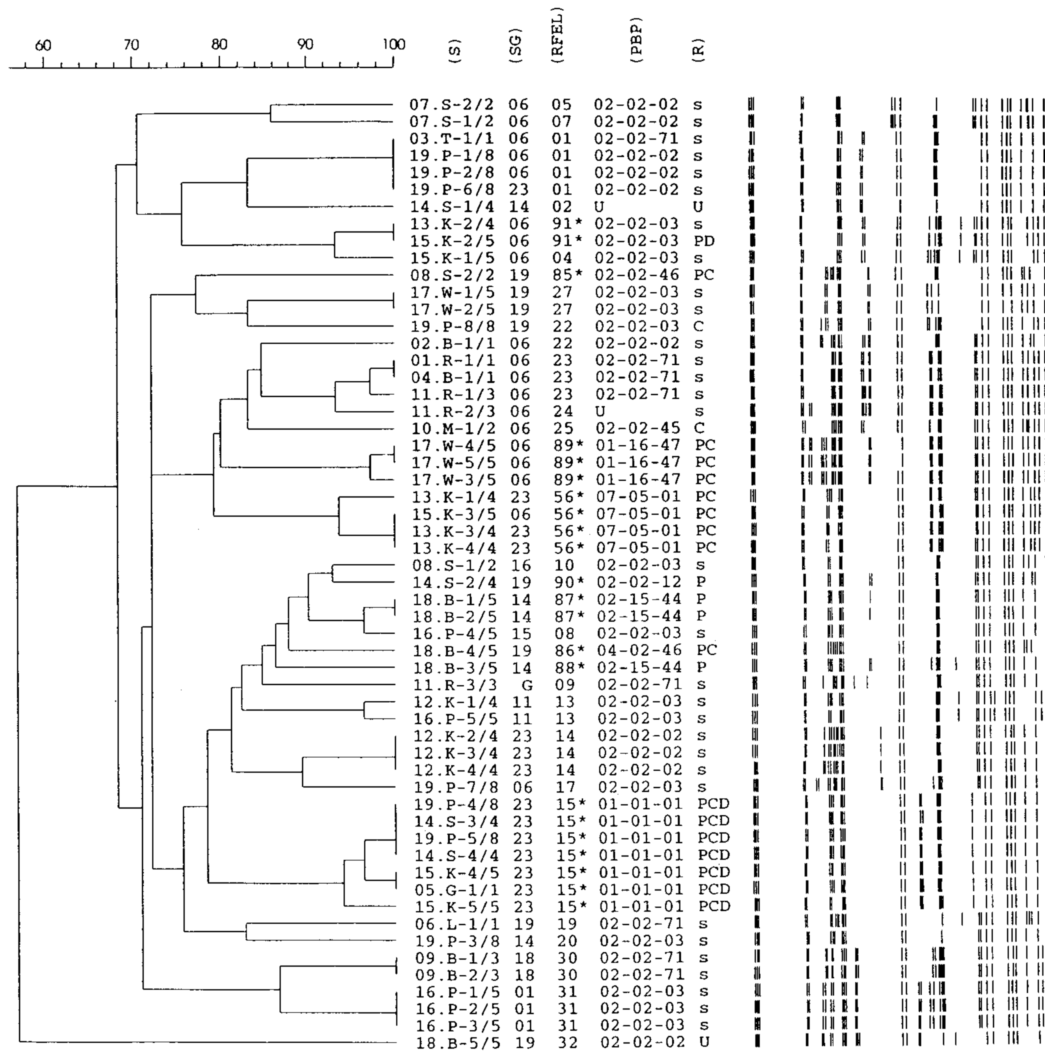

FIG. 1. Dendrogram of the RFEL banding patterns of 56 pneumococcal isolates from the nasopharynges of the children in the study. Subject (S), serogroup (SG), RFEL type (RFEL), PBP type (PBP), and resistance profile (R) are presented. The arbitrary cutoff value for identical RFEL types is 95%. RFEL numbering of penicillin-resistant strains (*) is in concordance with the numbering used by Hermans et al. (11). Since all RFEL type 15 strains (homology, >93%) demonstrated >95% homology with the pandemic clone 23F, they have identical RFEL codes. P, penicillin; C, co-trimoxazole; D, doxycycline; s, susceptible to all antibiotics tested; U, unknown.

BOX PCR genotyping was used to confirm the results for strains with identical RFEL types.

Among the 58 strains tested, serogroup 6 was encountered most frequently (34%). The high prevalence of serogroup 6 isolates among the group of young children studied is presumably the result of the poor immune response to capsular polysaccharide type 6 among children under the age of 2 years (15).

Eleven children were colonized with different types of pneumococcal strains. Repeated isolation of an identical RFEL type was observed for nine children. In all cases, the persistent colonization was confirmed by BOX PCR typing.

For child 19.P, from whom *S. pneumoniae* was isolated on eight occasions, the first two isolates and the sixth isolate displayed identical RFEL types, BOX PCR types, and PBP types. However, the first two isolates were capsular type 6, whereas the sixth isolate belonged to serogroup 23. Between the two periods of isolation of this particular strain, a strain of serogroup 23 resistant to multiple drugs was isolated from this child twice. This observation strongly suggests that the transfer of capsular genes occurred between these strains in child 19.P. The rationale behind this capsule switch, which took place from an antibiotic-resistant strain to an antibiotic-susceptible strain, is unclear. It is difficult to explain why a strain of serogroup 6 switches to serogroup 23. In this child, immune pressure to capsular type 6 is unlikely to have been the trigger for the capsular switch, since another pneumococcal strain of serogroup 6 was isolated at a later stage of the study. A plausible explanation for this event can be that the RFEL type 1 strain had become more virulent due to the acquisition of the type 23F capsule. Experiments with animals, in which mice are challenged intranasally with both versions of the RFEL type 1 strains, can assist with an investigation of this hypothesis.

RFEL type 56 was observed among two children. This type was isolated three times from child 13.K, and all isolates belonged to serogroup 23. Only one strain of RFEL type 56 was isolated from child 15.K. In contrast to the RFEL type 56 isolates from child 13.K, this isolate was of capsular type 6. These data suggest that horizontal transfer of capsular genes to

RFEL type 56 strains had occurred within this RFEL type during evolution.

Comparison of the RFEL patterns with those of 205 penicillin-resistant strains isolated in 15 countries and comprising 84 RFEL types (11) revealed 100% homology between the predominant RFEL type 15 and the previously described pandemic clone of serotype 23F (13, 17). Further analysis of the comparison with the 205 strains showed that the RFEL type 56 strains were identical to a strain isolated in Spain. However, the Spanish strain displayed a different serotype and a different PBP type, suggesting that multiple recombination events have taken place during the evolution of this RFEL type.

Resistance to antibiotics is frequently observed in isolates recovered from patients with acute otitis media and isolates from the nasopharynges. Rates of penicillin resistance ranging from 20 to 40% or higher have been described (20, 21). Thirty-nine percent of the pneumococcal isolates in this study had reduced susceptibility to penicillin. Most of the penicillin-resistant strains were resistant to trimethoprim-sulfamethoxazole (76%) or doxycycline (38%), or both drugs. This is in concordance with the results of other studies that have also found high rates of coresistance among *S. pneumoniae* isolates (11, 12). The high percentage of coresistance to penicillin and trimethoprim-sulfamethoxazole suggests that the cotransfer of penicillin resistance genes and trimethoprim resistance genes frequently occurs.

PBP genotyping demonstrated that the predominant PBP type among the penicillin-resistant strains was 1-1-1. This PBP type was uniquely associated with the highly penicillin-resistant and multiple drug-resistant strains of RFEL type 15, which were observed among four children. As described previously (13), this PBP genotype is highly prevalent among penicillin-resistant pneumococci and is predominantly observed in strains of the pandemic clones 23F and 9V. Four intermediately resistant isolates recovered from two children were PBP type 7-5-1. Strains of RFEL types 87 and 88 that were isolated from child 18.B both harbored PBP type 2-15-44, the PBP gene of which codes for an intermediate level of penicillin resistance. Although RFEL types 87 and 88 had only 85% homology, the BOX PCR types of the strains of these RFEL types were identical, indicating that these strains are genetically highly related. Penicillin-susceptible strains were invariably of PBP types 2-2-2, 2-2-3, and 2-2-71. Only one strain, a PBP type 2-2-3 strain, was intermediately resistant to penicillin (MIC, 0.5 μl/ml). This is the first pneumococcal strain among more than 100 genetically heterogeneous strains of PBP type 2-2-3 which is not susceptible to penicillin (22). Since this penicillin-resistant phenotype was not detected by PBP genotyping, we presume that a restricted number of point mutations in one of the PBP genes is responsible for the decrease in penicillin susceptibility in this particular strain. DNA sequencing of the PBP genes should reveal these mutations.

Since only one colony from each primary culture was used for analysis, we cannot be certain whether children were colonized with multiple different cocolonizing strains. To detect cocolonizing strains, one should pick various colonies from the primary culture of the nasopharyngeal swab specimen. In this way, the changes in nasopharyngeal colonization and events such as horizontal transfer of capsular genes and PBP genes can be studied in greater detail. Such a study is being undertaken in our laboratory.

Considering the capsular types that are to be included in the new conjugate vaccines (2, 5), serogroups 6, 14, 19, and 23 accounted for 81% of the pneumococcal isolates in the present study. The use of these conjugate vaccines might lead to an increasing prevalence of capsular types that are not included in these vaccines either by reinfection or by in vivo horizontal transfer of capsular genes. Although the capsular transfer observed in child 19.P occurred between two types covered by the vaccine, frequent horizontal transfer of capsular genes within individuals of the target group remains a potential threat for vaccination strategies in the future (11). In order to investigate the relevance of these observations, large-scale molecular epidemiological follow-up studies need to be performed with individuals who will receive the new conjugate vaccines in the near future.

REFERENCES

1. **Barnes, D. M., S. Whittier, P. H. Gilligan, S. Soares, A. Tomasz, and F. W. Henderson.** 1995. Transmission of multidrug-resistant serotype 23F *Streptococcus pneumoniae* in group day care: evidence suggesting capsular transformation of the resistant strain *in vivo*. J. Infect. Dis. **171:**890–896.
2. **Butler, J. C., R. F. Breiman, H. B. Lipman, J. Hofmann, and R. R. Facklam.** 1995. Serotype distribution of *Streptococcus pneumoniae* infections among preschool children in the United States, 1978–1994: implications for development of a conjugate vaccine. J. Infect. Dis. **171:**885–889.
3. **Caputo, G. M., M. Singer, S. White, and M. R. Weitekamp.** 1993. Infections due to antibiotic-resistant gram-positive cocci. J. Gen. Intern. Med. **8:**626–634.
4. **Coffey, T. J., C. G. Dowson, M. Daniels, J. Zhou, C. Martin, B. G. Spratt, and J. M. Musser.** 1991. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. Mol. Microbiol. **5:**2255–2260.
5. **Dagan, R., R. Melamed, M. Muallem, L. Piglansky, and P. Yagupsky.** 1996. Nasopharyngeal colonization in southern Israel with antibiotic-resistant pneumococci during the first 2 years of life: relation to serotypes likely to be included in pneumococcal conjugate vaccines. J. Infect. Dis. **174:**1352–1355.
6. **Dowson, C. G., A. Hutchison, and B. G. Spratt.** 1989. Extensive re-modelling of the transpeptidase domain of penicillin-binding protein 2B of a penicillin-resistant South African isolate of *Streptococcus pneumoniae*. Mol. Microbiol. **3:**95–102.
7. **Duffy, L. C., H. Faden, R. Wasielewski, J. Wolf, and D. Krystofik.** 1997. Exclusive breastfeeding protects against bacterial colonization and day care exposure to otitis media. Pediatrics **100:**E7.
8. **Facklam, R. R., and J. A. Washington.** 1991. *Streptococcus* and related catalase-negative gram-positive cocci, p. 238–257. *In* A. Balows, W. J. Hausler, Jr., R. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
9. **Faden, H., L. Duffy, R. Wasielewski, J. Wolf, D. Krystofik, Y. Tung, and Tonawanda/Williamsville Pediatrics.** 1997. Relationship between nasopharyngeal colonization and the development of otitis media in children. J. Infect. Dis. **175:**1440–1445.
10. **Gray, B. M., G. M. Converse, and H. C. Dillon, Jr.** 1980. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. J. Infect. Dis. **142:**923–933.
11. **Hermans, P. W. M., M. Sluijter, S. Dejsirilert, N. Lemmens, K. Elzenaar, A. van Veen, W. H. F. Goessens, and R. de Groot.** 1997. Molecular epidemiology of drug-resistant pneumococci: towards an international approach. Microb. Drug Resist. **3:**243–251.
12. **Hermans, P. W. M., M. Sluijter, K. Elzenaar, A. van Veen, J. J. M. Schonkeren, F. M. Nooren, W. J. van Leeuwen, A. J. de Neeling, B. van Klingeren, H. A. Verbrugh, and R. de Groot.** 1997. Penicillin-resistant *Streptococcus pneumoniae* in The Netherlands: results of a 1-year molecular epidemiologic survey. J. Infect. Dis. **175:**1413–1422.
13. **Hermans, P. W. M., M. Sluijter, T. Hoogenboezem, H. Heersma, A. van Belkum, and R. de Groot.** 1995. Comparative study of five different DNA fingerprint techniques for molecular typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. **33:**1606–1612.
14. **Homoe, P., J. Prag, S. Farholt, J. Henrichsen, A. Hornsleth, M. Kilian, and J. S. Jensen.** 1996. High rate of nasopharyngeal carriage of potential pathogens among children in Greenland: results of a clinical survey of middle-ear disease. Clin. Infect. Dis. **23:**1081–1090.
15. **Lindberg, K., A. Freijd, B. Rynnel-Dagoo, and L. Hammarstrom.** 1993. Anti pneumococcal antibody activity in nasopharyngeal secretions in healthy adults and children. Acta Otolaryngol. (Stockholm) **113:**673–678.
16. **Martin, B., O. Humbert, M. Camara, E. Guenzi, J. Walker, T. Mitchell, P. Andrew, M. Prudhomme, G. Alloing, R. Hakenbeck, et al.** 1992. A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res. **20:**3479–3483.
17. **Munoz, R., T. J. Coffey, M. Daniels, C. G. Dowson, G. Laible, J. Casal, R. Hakenbeck, M. Jacobs, J. M. Musser, B. G. Spratt, et al.** 1991. Intercontinental spread of a multiresistant clone of serotype 23F *Streptococcus pneumoniae*. J. Infect. Dis. **164:**302–306.

18. **Murray, P. R., E. J. Baron, M. A. Pfaller, F. C. Tenover, R. H. Yolken (ed.).** 1995. Manual of clinical microbiology. American Society for Microbiology. Washington, D.C.
19. **National Committee for Clinical Laboratory Standards.** 1995. Performance standards for antimicrobial susceptibility testing; sixth informational supplement. NCCLS document M100-S6. National Committee for Clinical Laboratory Standards, Wayne, Pa.
20. **Ostroff, S. M., L. H. Harrison, N. Khallaf, M. T. Assaad, N. I. Guirguis, S. Harrington, M. el-Alamy, and The Antimicrobial Resistance Surveillance Study Group.** 1996. Resistance patterns of *Streptococcus pneumoniae* and *Haemophilus influenzae* isolates recovered in Egypt from children with pneumonia. Clin. Infect. Dis. **23:**1069–1074.
21. **Pons, J. L., M. N. Mandement, E. Martin, C. Lemort, M. Nouvellon, E. Mallet, and J. F. Lemeland.** 1996. Clonal and temporal patterns of nasopharyngeal penicillin-susceptible and penicillin-resistant *Streptococcus pneumoniae* stains in children attending a day-care center. J. Clin. Microbiol. **34:** 3218–3222.
22. **Sluijter, M.** 1998. Unpublished observations.
23. **van Belkum, A., M. Sluijter, R. de Groot, H. Verbrugh, and P. W. M. Hermans.** 1996. Novel BOX repeat PCR assay for high-resolution typing of *Streptococcus pneumoniae* strains. J. Clin. Microbiol. **34:**1176–1179.
24. **van Steenbergen, T. J., S. D. Colloms, P. W. M. Hermans, J. de Graaff, and R. H. Plasterk.** 1995. Genomic DNA fingerprinting by restriction fragment end labeling. Proc. Natl. Acad. Sci. USA **92:**5572–5576.
25. **Zenni, M. K., S. H. Cheatham, J. M. Thompson, G. W. Reed, A. B. Batson, P. S. Palmer, K. L. Holland, and K. M. Edwards.** 1995. *Streptococcus pneumoniae* colonization in the young child: association with otitis media and resistance to penicillin. J. Pediatr. **127:**533–537.