Emergence of Penicillin-Nonsusceptible *Streptococcus pneumoniae* Clones Expressing Serotypes Not Present in the Antipneumococcal Conjugate Vaccine

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Background. Penicillin-nonsusceptible *Streptococcus pneumoniae* isolates are confined mainly to a few serogroups. Capsular transformation may serve as a mechanism for spreading antibiotic resistance to new serotypes.

Methods. Antibiogram and molecular typing, by pulsed-field gel electrophoresis (PFGE), were performed on 46 nasopharyngeal and middle ear fluid (MEF) isolates expressing serotype 11A, 45 MEF isolates expressing serotype 15B/C (recovered during 1998–2003 from Israeli children <5 years old), and 57 MEF isolates expressing serotype 19F (recovered during 1998–2001 from Costa Rican children <7.5 years old).

Results. PFGE patterns showed that 49 (86%) of 57 serotype 19F isolates and 19 (41%) of 46 serotype 15B/C isolates were closely related. The vast majority of these isolates (80% of serotype 19F and 100% of serotype 15B/C isolates) were nonsusceptible to penicillin. Multilocus sequence typing (MLST) data show that the serotype 15B/C isolates belonged to the ST346 cluster, whereas the serotype 19F isolates were a single-locus variant of ST346. For serotype 11A isolates, PFGE patterns and MLST analysis showed that 8 (80%) of the 10 penicillin-nonsusceptible isolates belonged to a single clone—namely, ST156—which was identical to the international Spain^{9V}-3 clone.

Conclusions. Penicillin-nonsusceptible pneumococcal clones of serotypes not related to those included in the 11-valent conjugate vaccines may derive from capsular transformation of vaccine-related serotypes. Of particular concern was the detection of serotype 11A variants of the successful international Spain^{9V}-3 clone. This phenomenon, although seemingly rare at present, can have implications for the long-term effectiveness of the conjugate vaccines.

Pneumococcal capsular polysaccharide is a major virulence factor and is a target for protective immunity involved with evasion of the host immune system [1]. For reasons that have not been fully elucidated, penicillin-nonsusceptible *Streptococcus pneumoniae* isolates are most frequently encountered in only a few of the 90 different serotypes that have been identified [2–5]. Multivalent pneumococcal conjugate vaccines containing 7–11 serotypes have been developed to protect in-

fants from pneumococcal disease caused by the common childhood strains [6]. Several studies have reported that administration of pneumococcal conjugate vaccines reduced nasopharyngeal carriage of pneumococcal serotypes included in the vaccine (vaccine type [VT] serotypes) [7–9]. However, replacement of VT serotypes and, sometimes, serotypes antigenically related to VT serotypes (VT related) by non-VT serotypes, in the nasopharynx and middle ear fluid (MEF), has been reported [7–12].

In a recent study [13], we examined the prevalence of non-VT strains recovered from MEF of children <3 years old from southern Israel with acute otitis media (AOM). Approximately 20% (477/2467) of all AOM episodes were caused by serotypes not included in and not immunologically related to the 11-valent conjugate vaccine. Of these episodes, more than one-third were due to organisms belonging to only 4 serotypes: 35B,

Table 1. *Streptococcus pneumoniae* isolates included in the present study.

Serotype	No. of isolates	Source	Country of isolation	Year(s) of isolation	Age of children, range, months
14	1	NP	Israel	1997	21
9V	1	NP	Israel	1997	33
9V[a]	2	ND	United States	ND	ND
11A	20	NP	Israel	1999–2003	6–60
11A	26	MEF	Israel	1999–2003	6–60
15B/C[b]	45	MEF	Israel	1998–2001	2–24
19F	57	MEF	Costa Rica	1999–2001	4–90

NOTE. MEF, middle ear fluid; ND, no data; NP, nasopharynx.

[a] Provided by A. Tomasz, The Rockefeller University, New York.

[b] Serotypes 15B and 15C are known to interconvert in vitro and in vivo by the addition of an acetyl group to the capsular polysaccharide of serotype 15C, to form an *O*-acetylated 15B variant, and, thus, are regarded, at present, as 1 serotype (15B/C) [16].

33F, 21, and 15B/C. Most isolates belonging to these serotypes were of clonal origin, and most were nonsusceptible to penicillin. The high prevalence of these clones, even before the introduction of the pneumococcal conjugate vaccines, raises the potential of replacement with non-VT antibiotic-resistant clones after initiation of universal vaccination with pneumococcal conjugate vaccines.

The ability of pneumococci to undergo genetic transformation has been demonstrated both in vitro and in vivo [14, 15]. Acquisition of a new serotype may provide the transformant with an advantage against the immune system of the host. Thus, in vivo capsular transformation may serve as a mechanism for spreading antibiotic resistance to new serotypes—that is, serotypes other than the currently dominant "pediatric" serotypes targeted by the conjugate vaccines—changing the clinical epidemiology of pneumococcal disease.

The majority of penicillin-nonsusceptible clones are of VT serotypes, and, although serotype variants of the major nonsusceptible clones have been described, they have mostly involved changes to other VT serotypes. However, in vivo capsular transformation events in which a serotype 23F Spanish/US clone was the recipient of non-VT capsular genes 3 and 9N have been described elsewhere [14]. In the present study, we describe a penicillin-nonsusceptible non-VT clone of serotype 11A that was recovered from children from southern Israel and has been shown to be a serotype variant of the international Spain[9V]-3 clone. We speculate that the new clone resulted from spontaneous transformation events in which the non-VT capsular genes were transferred to a penicillin-nonsusceptible strain of the common pediatric capsular type, thereby causing the spread of resistance genes to a non-VT serotype. The second penicillin-nonsusceptible non-VT clone of serotype 15B/C described here (seen in Norway during 1995) was recovered from children from southern Israel and was found to be very similar in genotype to a penicillin-nonsusceptible Costa Rican VT clone of serotype 19F. However, the

directionality of the switch between the 15B/C and 19F clones is not clear.

MATERIALS AND METHODS

Bacterial isolates. The pneumococcal isolates included in the present study are listed in table 1. Pneumococcal isolates were characterized by inhibition with optochin and by a positive slide agglutination test (Phadebact; Pharmacia Diagnostics).

Antibiotic susceptibility testing. Testing of isolates for susceptibility to penicillin, erythromycin, tetracycline, chloramphenicol, clindamycin, and trimethoprim-sulfamethoxazole (TMP/SMX) was performed by the disk-diffusion method of Bauer and Kirby, following NCCLS recommendations [17]. Isolates exhibiting an inhibition zone with a diameter ≤19 mm around a 1-μg oxacillin disk were further tested for susceptibility to penicillin, by use of the E-test (AB Biodisk), following the manufacturer's instructions [18]. Isolates with a penicillin MIC <0.1 μg/mL were defined as susceptible to penicillin, and those with an MIC ≥0.1 μg/mL were defined as nonsusceptible to penicillin.

Serogrouping and serotyping. Serogrouping and serotyping of *S. pneumoniae* were done by the quellung reaction, by use of antisera from Statens Serum Institute of Copenhagen, Denmark [19].

Pulsed-field gel electrophoresis (PFGE). Chromosomal DNA fragments, generated by *Sma*I and *Apa*I digestion, were prepared and analyzed as described elsewhere [20]. A contour-clamped homogenous electric field DRIII apparatus (Bio-Rad Laboratories) was used for running the gels. Running conditions were 11.3°C at 200V, with an initial pulse time of 5 s increased to 35 s over the course of 23 h. Gels were stained with ethidium bromide and photographed. Interpretation of strain relatedness, on the basis of PFGE patterns, was performed according to current consensus criteria [21].

Multilocus sequence typing (MLST). Pneumococcal iso-

lates were unambiguously characterized by MLST, as described by Enright and Spratt [22]. The sequences (alleles) at each locus were compared with those at the MLST Web site (http://www.mlst.net) and were assigned allele numbers if they corresponded to sequences already submitted to the MLST database of pneumococci. The allelic profiles of isolates (the allele numbers at the 7 loci) were compared with those at the MLST Web site, and sequence types were assigned.

RESULTS

Comparison between serotype 11A isolates and the international Spain^{9V}-3 clone. A total of 46 isolates expressing serotype 11A, recovered from MEF ($n = 26$) and the nasopharynx ($n = 20$) of Israeli children, were included in the present study. PFGE patterns generated by *Sma*I digestion (figure 1, lanes *1–8*) and *Apa*I digestion (figure 1, lanes *13–20*) showed that 8 (80%) of the 10 penicillin-nonsusceptible isolates belonged to the same clone. The PFGE pattern of this clone was compared with those of the international Spain^{9V}-3 clone [23], which were generated from 2 isolates recovered from children attending day-care centers in Israel and from 2 isolates from the United States (provided by A. Tomasz, The Rockefeller University, NY). The *Sma*I and *Apa*I PFGE patterns of the penicillin-nonsusceptible serotype 11A organisms were very similar to those of the international Spain^{9V}-3 clone [23] (max-

imum difference, 2 band) (figure 1, lanes *9–12* and *21–24*), indicating that these isolates are very closely related and can be considered to be members of the same clone.

In contrast, the other 38 serotype 11A isolates, of which 2 were nonsusceptible to penicillin and 36 were susceptible to penicillin, showed 5 PFGE patterns with 3 major clones (figure 2), none of which was similar to the pattern of the penicillin-nonsusceptible clone. Four representative isolates of the serotype 11A clone were also characterized by MLST (table 2). The allelic profile of this clone was identical to that of the international Spain^{9V}-3 clone (ST156), indicating that the penicillin-nonsusceptible serotype 11A clone is a capsular-switch variant of the international Spain^{9V}-3 clone.

The antimicrobial susceptibility profiles of the 8 penicillin-nonsusceptible isolates expressing serotype 11A were similar to that of the international Spain^{9V}-3 clone (table 3). Penicillin MIC values were in the range of 0.25–1.00 μg/mL, and 7 (88%) of 8 isolates were resistant to TMP/SMX. All these penicillin-nonsusceptible serotype 11A isolates were susceptible to erythromycin, tetracycline, chloramphenicol, and clindamycin, as was the international Spain^{9V}-3 clone.

Comparison between serotype 19F and serotype 15B/C isolates. A total of 57 isolates expressing serotype 19F, recovered from MEF of Costa Rican children, were included in the present study. PFGE patterns generated by *Sma*I and *Apa*I digestion
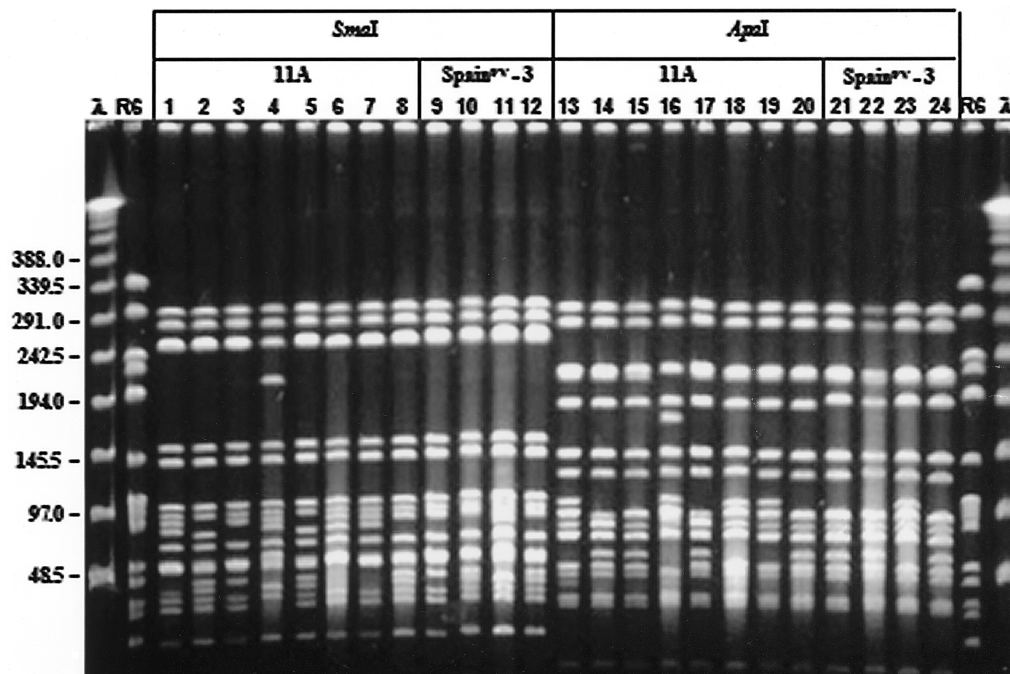

Figure 1. Pulsed-field gel electrophoresis patterns of chromosomal *Sma*I digests *(lanes 1–12)* and *Apa*I digests *(lanes 13–24)* of penicillin-nonsusceptible serotype 11A *Streptococcus pneumoniae* isolates and the international Spain^{9V}-3 clone. Lanes *1–12, Sma*I digests of 8 serotype 11A isolates *(lanes 1–8)* and 4 isolates belonging to the international Spain^{9V}-3 clone *(lanes 9–12)*; lanes *13–24, Apa*I digests of 8 serotype 11A isolates *(lanes 13–20)* and 4 isolates belonging to the international Spain^{9V}-3 clone *(lanes 21–24)*. λ, lambda ladder; R6, *S. pneumoniae* reference strain used as a molecular weight marker.
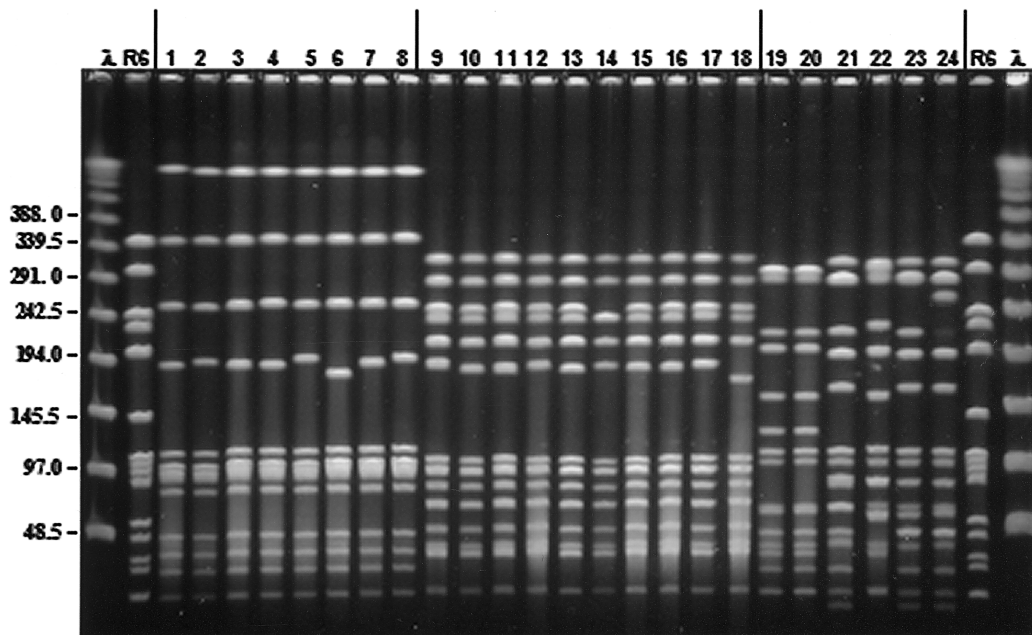
Figure 2. Pulsed-field gel electrophoresis patterns of chromosomal *Sma*I digests of selected penicillin-susceptible serotype 11A *Streptococcus pneumoniae* isolates. *Lanes 1–24*, 3 different patterns of penicillin-susceptible serotype 11A isolates. λ, lambda ladder; R6, *S. pneumoniae* reference strain used as a molecular weight marker.

showed that 49 (86%) of 57 isolates belonged to the same clone (6 representative patterns are shown in figure 3, *lanes 7–12* and *19–24*). The PFGE patterns of these serotype 19F isolates were similar to those of 19 penicillin-nonsusceptible isolates expressing serotype 15B/C that were recovered from MEF of children from southern Israel [13] (6 representative patterns are shown in figure 3, *lanes 1–6* and *13–18*). In contrast, 26 penicillin-susceptible serotype 15B/C isolates showed 8 different PFGE patterns [13], none of which was similar to that of the penicillin-nonsusceptible 15B/C clone.

Four representative serotype 19F and 15B/C isolates (table 2) were further characterized by MLST. Isolates selected for characterization by MLST were those belonging to the major clone of each serotype but showing the greatest difference in their PFGE patterns. The MLST data revealed that the serotype 15B/C isolates were a single clone with the same allelic profile as that of a previously characterized serotype 15B/C strain from Norway (ST346; according to http://www.mlst.net). The MLST profile of the serotype 19F strain was novel but was very similar to that of ST346, differing at only 1 of 7 MLST loci (*rec*P). The serotype 19F strain has been entered into the MLST database and designated as ST1203.

The antimicrobial susceptibility profiles of the penicillin-nonsusceptible serotype 19F and 15B/C isolates were also very similar (table 3). Of the 49 serotype 19F Costa Rican isolates belonging to the same clone, 39 (80%) were intermediately resistant to penicillin (0.125 ≤ MIC ≤ 0.5 μg/mL), and 33 (67%) were resistant to TMP/SMX. Rates of susceptibility to

erythromycin, tetracycline, chloramphenicol, and clindamycin were 94%, 98%, 98%, and 96%, respectively. Likewise, all 19 serotype 15B/C isolates were intermediately resistant to penicillin (0.25 ≤ MIC ≤ 0.5 μg/mL), and only 3 (16%) were also resistant to TMP/SMX. All 19 isolates were susceptible to erythromycin, tetracycline, chloramphenicol, and clindamycin.

DISCUSSION

In early 2000, a 7-valent protein-polysaccharide pneumococcal conjugate vaccine (Prevnar; Wyeth-Ledele Vaccines) was licensed for use in infants and young children in the United States. Controlled clinical trials have shown that, in infants, the vaccine is highly efficacious against invasive disease [24] and somewhat efficacious against AOM [6] and pneumonia [25]. Furthermore, postlicensure data from the United States show an extensive reduction in invasive diseases caused by VT serotypes [26, 27] and by antibiotic-nonsusceptible *S. pneumoniae* [28].

Vaccination tends to cause a shift toward carriage of non-VT strains [7–9]. In 2 studies performed in Finland [6, 29] with 2 different 7-valent pneumococcal conjugate vaccines, administration of the vaccine was associated with a 27%–33% increase in the rate of AOM caused by non-VT strains. Postlicensure data from the United States show the same trend [12, 30]. In contrast, for invasive infections, the effect seems, so far, to be minimal [26], presumably because replacing non-VT strains has a relatively low ability to cause invasive disease.

The degree to which genetic transformation of the prevalent

Table 2. Sequence typing of pneumococcal isolates.

Strain	Year of isolation	Source	Country of isolation	Serotype	Lane no. in PFGE gel[a]		Allele no.							Sequence type
					*Sma*I	*Apa*I	*aro*E	*gdh*	*gki*	*rec*P	*spi*	*xpt*	*ddl*	
PM133	2002	NP	Israel	11A	3	15	7	11	10	1	6	8	1	156
C99NP167	2000	NP	Israel	11A	1	13	7	11	10	1	6	8	1	156
C99NP324	2000	NP	Israel	11A	7	19	7	11	10	1	6	8	1	156
C0621	2000	NP	Israel	11A	8	20	7	11	10	1	6	8	1	156
OM525	2000	MEF	Israel	15B/C	4	16	10	41	47	16	6	14	2	346
OM7	1999	MEF	Israel	15B/C	1	13	10	41	47	16	6	14	2	346
OM1259	2001	MEF	Israel	15B/C	5	17	10	41	47	16	6	14	2	346
OM1087	2001	MEF	Israel	15B/C	6	18	10	41	47	16	6	14	2	346
62597AG99	1999	MEF	Costa Rica	19F	7	19	10	41	47	63	6	14	2	1203
62573AG00	2000	MEF	Costa Rica	19F	8	20	10	41	47	63	6	14	2	1203
63AG99	1999	MEF	Costa Rica	19F	9	21	10	41	47	63	6	14	2	1203
64AG99	1999	MEF	Costa Rica	19F	10	22	10	41	47	63	6	14	2	1203

NOTE. *aro*E, gene encoding shikimate dehydrogenase; *ddl,* gene encoding D-ala-D-ala ligase; *gdh,* gene encoding glucose-6-phosphate dehydrogenase; *gki,* gene encoding glucose kinase; MEF, middle ear fluid; NP, nasopharynx; PFGE, pulsed-field gel electrophoresis; *spi,* gene encoding signal peptidase I; *xpt,* gene encoding xanthine phosphotransferase.

[a] Lanes refer to PFGE patterns presented in figure 1, for serotype 11A, and in figure 3, for serotypes 15B/C and 19F.

VT strains will contribute to this shift to non-VT serotypes is unknown. The only way to detect such events is by genotypic analysis of the strains. PFGE is considered to be a technique that is well suited to the analysis of large numbers of isolates. Using this method, we found 2 clusters expressing non-VT serotypes with a genetic background closely related to that of 2 VT clones. This was confirmed by MLST analysis of 7 internal ∼500-bp fragments from housekeeping genes.

The first clone was found in a cluster of 8 isolates expressing serotype 11A that were recovered during 2000–2003 from the nasopharynx (6/8 [75%]) and MEF (2/8 [25%]) of young Israeli children. This clone was more common among Bedouin children from southern Israel (7/8 [88%]), who experienced standards of living resembling those in developing populations, than among Jews (1/8 [13%]). All the isolates of this clone were nonsusceptible to penicillin, and most (7/8 [88%]) were also resistant to TMP/SMX. The PFGE patterns of these isolates, generated by *Sma*I and *Apa*I digestion, were almost indistinguishable (maximum difference, 2 band) from those of the penicillin-resistant serotype 9V/14 international clone (Spain[9V]-3) [23], which was first identified in Spain and France. The allelic profile of this clone was identical to that of the international Spain[9V]-3 clone (ST156), indicating that the serotype 11A clone is a serotype variant of the international Spain[9V]-3 clone. This clone has become widely disseminated throughout the world, including in Israel [31], and is shared by strains expressing different capsular types, mainly of serogroups 9, 14, and 19.

The second non-VT clone, expressing serotype 15B/C, was recovered during 1998–2001 from MEF of young Israeli children [13]. This clone was more common among Bedouin children from southern Israel (16/19 [84%]) than among Jews (3/19 [16%]). The PFGE patterns of this clone, generated by *Sma*I

and *Apa*I digestion, show close relatedness to the patterns produced by these enzymes for a serotype 19F clone, which was recovered during 1999–2001 from MEF of Costa Rican children [32]. MLST data reveal that the 15B/C isolates belonged to the ST346 cluster and were very similar to the new Costa Rican 19F clone, differing only at the *rec*P locus. The vast majority of isolates belonging to these clusters were nonsusceptible to penicillin and TMP/SMX but were susceptible to 4 other antibiotic drugs tested.

Several examples of strains sharing the same genetic background but having different serotypes have been reported [1, 14, 15, 33, 34]. Molecular studies have shown that such changes in serotype occur by recombinational events that replace the capsular biosynthetic genes of a recipient pneumococcus with the corresponding genes from a donor pneumococcus of a different serotype. These examples have been primarily limited

Table 3. Antimicrobial susceptibility pattern of isolates belonging to the 3 major clones of serotypes 11A, 15B/C, and 19F.

	Serotype		
Pattern	11A (*n* = 8)	15B/C (*n* = 19)	19F (*n* = 49)
Penicillin MIC, range, µg/mL	0.25–1.00	0.25–0.5	0.015–0.5
Nonsusceptible to			
Penicillin	100	100	80
TMP/SMX	88	16	67
Erythromycin	0	0	6
Tetracycline	0	0	2
Chloramphenicol	0	0	2
Clindamycin	0	0	4

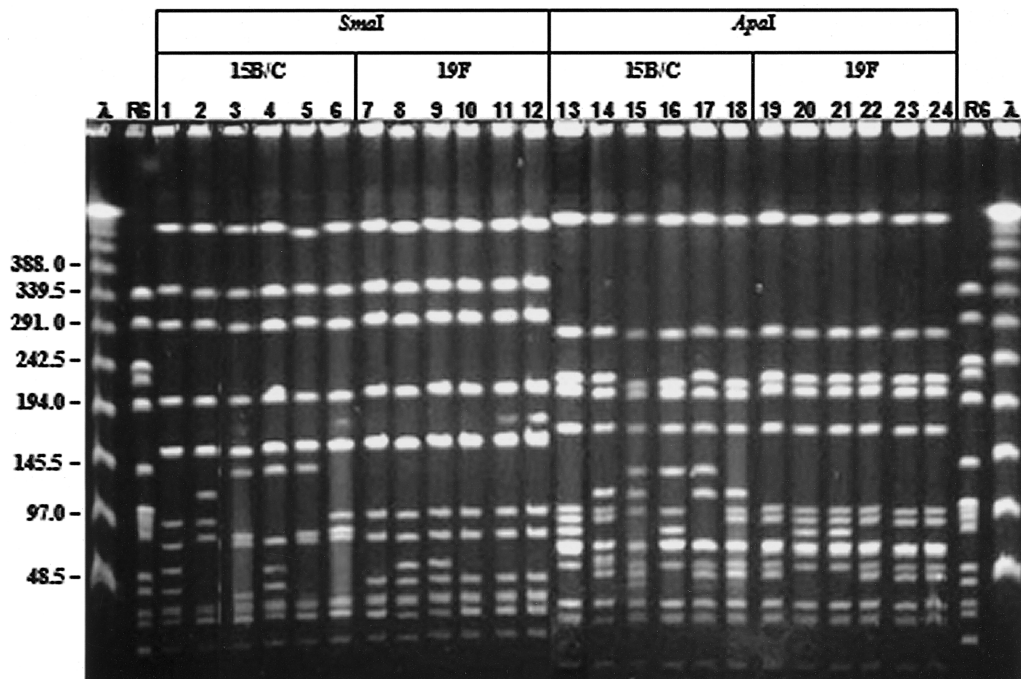**NOTE.** Data are percentages, unless otherwise noted. TMP/SMX, trimethoprim-sulfamethoxazole.

Figure 3. Pulsed-field gel electrophoresis patterns of chromosomal *Sma*I digests *(lanes 1–12)* and *Apa*I digests *(lanes 13–24)* of selected penicillin-nonsusceptible serotype 15B/C and serotype 19F *Streptococcus pneumoniae* isolates. *Lanes 1–12, Sma*I digests of 6 serotype 15B/C isolates *(lanes 1–6)* and 6 serotype 19F isolates *(lanes 7–12); lanes 13–24, Apa*I digests of 6 serotype 15B/C isolates *(lanes 13–18)* and 6 serotype 19F isolates *(lanes 19–24)*. λ, lambda ladder; R6, *S. pneumoniae* reference strain used as a molecular weight marker.

to serogroups 6, 9, 14, 19, and 23, which are included in the conjugate vaccines, because these serogroups are the most common and are carried for longer periods of time in the nasopharynx of infants and young children [15, 16]. McEllistrem et al. [12] previously reported the detection, by PFGE, of possible capsular switching among VT and non-VT strains—namely, serogroups 19-15 and 6-14-35. Nesin et al. [14] described in vivo capsular transformation events in which the serotype 23F Spanish/US clone was the recipient of VT capsular genes 14 and 19F, as well as the non-VT capsular genes 3 and 9N. In the present study, we have presented a serotype 11A non-VT capsular-switch clone of the penicillin-nonsusceptible international Spain^9V-3 clone.

The appearance of clones with multiple serotypes is considered to be due to capsular transformation, but the frequency with which this phenomenon occurs is not clear. In a recent birth cohort study [16], molecular characterization by MLST was performed on 333 pneumococci recovered from 100 infants during the first 2 years of life. There was no evidence in these children of any changes of serotype mediated by recombinational replacement at the capsular locus during nasopharyngeal carriage. Thus far, only 4 examples of recognized serotype change in vivo have been published [14, 15, 33, 34], suggesting that this phenomenon is relatively rare. In the case of the 2 non-VT clones described here, we were not able to find any epidemiological data that link the strains of the various sero-

types. Therefore, we can only speculate that the mechanism responsible for this phenomenon is capsular switch.

Asymptomatic nasopharyngeal carriage of pneumococci is widely prevalent among young children. The most common serotypes are 6A, 6B, 9V, 14, 19F, 19A, and 23F [7]. These serotypes often show high rates of resistance to various antimicrobial agents. A second cluster of certain non-VT serotypes—such as 11A, 15B/C, 21, 33F, 35B—is less often carried and shows lower rates of resistance to antimicrobial agents [13, 35], whereas a third group of serotypes is rarely carried in the nasopharynx and shows extremely low rates of resistance to antimicrobial agents. The latter group includes serotypes 1, 5, and 7F, which appear to be more prevalent in invasive disease than in carriage [36, 37]. On the basis of the data presented in the present study, we speculate that non-VT strains, which are less prevalent in the nasopharynx, are less commonly exposed to antibiotic pressure than are VT strains, resulting in a lower rate of emergence of resistance to antibiotics. Similarly, as they are rarely carried in many populations, non-VT strains are infrequent donors of capsular genes to other strains. In some populations, particularly those in developing countries or particular groups within developed countries, non-VT strains may be more prevalent in the nasopharynx of children and would be more capable of transferring their capsular genes to other strains through recombination events, thus causing a change in the capsular type of the well-established VT strains.

An increased prevalence of carriage of non-VT strains may also be expected in populations in which conjugate vaccines are used, leading to increased exposure of these strains to antibiotic pressure and increased opportunities for them to transfer capsular genes to VT strains. On the other hand, the reduction in carriage of VT strains caused by vaccination could reduce the opportunity of the non-VT strains to donate their capsule to VT strains and, by this, reduce the opportunity of the emergence of antibiotic-nonsusceptible non-VT strains.

The existence in Israel (a population in which conjugate vaccines have not been introduced) of serotype 11A variants of the international Spain[9V]-3 clone, one of the most successful antibiotic-nonsusceptible pneumococcal clones, is of concern because serotype replacement after introduction of a conjugate vaccine would be expected to increase the prevalence of such a clone. Capsular switching of "international resistant clones" expressing pediatric serotypes (9V/14 and 19F) to non-VT serotypes, although rare at present, can have implications for the efficacy of the conjugate vaccine. The appearance and increased prevalence of non-VT variants of the major antibiotic-nonsusceptible clones may be of minor consequence for invasive disease if these variants have a relatively low invasiveness, but a recent study suggests that non-VT strains may be as able as VT strains to cause AOM [38]. Future surveillance studies after mass vaccination will be required to detect the emergence of non-VT variants of the major resistant clones and to understand their impact on pneumococcal disease.

Acknowledgment

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