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Diversity and Antibiotic Resistance among Nonvaccine Serotypes of *Streptococcus pneumoniae* Carriage Isolates in the Post–Heptavalent Conjugate Vaccine Era

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Background. In response to the selective pressure of pneumococcal conjugate vaccine, increased asymptomatic carriage of antibiotic-nonsusceptible nonvaccine serotypes (NVTs) has been observed. Possible mechanisms include de novo acquisition of resistance, serotype switching, introduction of new clones, and expansion of existing clones.

Methods. To investigate the process of increased antibiotic nonsusceptibility among replacing serotypes, we applied multilocus sequence typing to samples of 126 and 222 pneumococci collected in 2001 and 2004, respectively, from the nasopharynges of children <7 years of age in 16 Massachusetts communities.

Results. We found no evidence of penicillin resistance due to either serotype switching or de novo acquisition. Nonetheless, resistance increased through the expansion of previously recognized clones of NVTs, particularly in serotypes 19A, 15A, and 35B. In 19A, several unrelated clones increased in frequency, whereas, in the other 2 serotypes, single resistant lineages were responsible for the increased prevalence of resistant strains.

Conclusions. The decreased prevalence of antibiotic resistance with the introduction of heptavalent pneumococcal conjugate vaccine is likely to be partially eroded over time as vaccine-included serotypes are replaced by resistant clones of NVTs. The clinical significance of this will depend on the pathogenic potential of replacing clones to cause local (e.g., otitis media) or invasive disease.

Immunization with conjugate vaccine has proved successful in the prevention of invasive pneumococcal disease (IPD) due to *Streptococcus pneumoniae,* both in the targeted pediatric population [1] and among older

patients through a herd effect [2]. The vaccine currently in use (heptavalent pneumococcal conjugate vaccine [PCV7]) was designed to include the 7 pneumococcal serotypes responsible for 70%–80% of IPD [3]. Because antibiotic nonsusceptibility was largely concentrated in these serotypes, the vaccine was also predicted to be effective against 78% of penicillin-nonsusceptible pneumococci (PNSP) that causes IPD [4]. However, any vaccination program represents a new selective pressure on the pathogen in question, and serotype replacement, in which nonvaccine serotypes (NVTs) increase in prevalence as vaccine types disappear from the population, has been documented [5–7]. Although surveillance of invasive isolates continues [8], the primary reservoir of pneumococci in the community is asymptomatic carriage in the nasopharynges of children [9], and, hence, monitoring changes in pediatric carriage can provide early insight into postvaccine effects. The remaining

critical question is the effect that serotype replacement in these populations will have on both local (e.g., otitis media and sinusitis) and IPD, particularly with antibiotic-resistant strains.

The concentration of penicillin nonsusceptibility among serotypes included in the currently licensed vaccine is likely to be a reflection of the high prevalence of carriage of these serotypes in the prevaccine pediatric population. Because young children typically receive a large number of antibiotic prescriptions, strains carried by this age group were under the greatest selective pressure. In the postvaccine era, resistant clones of NVT strains may likewise have a relative advantage for establishing successful colonization and expansion. For example, our study of pneumococcal carriage in 16 Massachusetts communities in 2001 and 2004 demonstrated serotype replacement and an increase in PNSP among NVT strains from 8% to 25%, along with resistance to additional antibiotic classes [5].

This increase in resistance among NVT strains may be occurring in several ways: clones that were present before vaccination (in the community already or in another locale from which they were imported) could now be expanding into the niche vacated by the vaccine serotypes. Alternatively, there may be NVT clones that, in the process of expansion, have acquired resistance de novo. Finally, because of the high frequency of recombination in this species, PNSP clones of vaccine serotypes may have acquired a new capsule through genetic exchange of the capsular locus—a process known as serotype switching [10]. Serotype switching raises the possibility that, over time, we could face the same resistant clones from the prevaccine era, except with new serotypes.

To address this, we used multilocus sequence typing (MLST) to determine the sequence types (STs) of previously collected carriage isolates from young children in 16 Massachusetts communities [5] and compared these with a database of >2000 pneumococcal STs from around the world, including internationally distributed clones of antibiotic-resistant pneumococci identified by the Pneumococcal Molecular Epidemiology Network (PMEN) [11–14]. By using MLST, we sought to determine (1) the population structure of *S. pneumoniae* under selective pressure of universal PCV7 immunization and (2) whether emerging nonvaccine strains represent expansion of preexisting clones in these communities, known clones imported from other locales, or examples of serotype switching.

MATERIALS AND METHODS

Data collection. Carried pneumococcal strains were collected by nasopharyngeal swab between 13 March and 11 May 2001 and between 17 November 2003 and 16 April 2004 from children <7 years of age attending pediatric and family medical practices for well-child or sick visits in the same 16 Massachusetts communities. Practices sampled in 2003–2004 were a subset (23/31 practices) of those sampled in 2001. Sampling and processing methods were identical in both periods [5]. Vaccine penetration (receipt of at least 1 PCV7 dose) increased from 60% in 2001 to 96% in 2004 among children <12 months of age and from 38% to 79% among children at least 12 months of age. Samples were processed for *S. pneumoniae* growth, antibiotic susceptibility, and serotype, as described elsewhere [5]. Clinical and Laboratory Standards Institute (CLSI) susceptibility breakpoints were used. Strains were maintained as glycerol stocks at −80°C, and DNA was purified using DNeasy Tissue Kits (Qiagen). After this work, and in response to a growing consensus, we have considered 19A as an NVT. Although this serotype is part of the same serogroup as 19F, it is clear that the vaccine produces limited functional antibody response to 19A and has little, if any, efficacy against 19A carriage.

MLST. STs of isolates were determined by MLST, as described elsewhere [15]. Sequences of each of the 7 gene fragments used in the pneumococcal MLST scheme were obtained on both DNA strands by use of an Applied Biosystems 3700 capillary sequencer and were analyzed by use of STARS (available at: http://www.mlst.net or http://www.molbiol.ox.ac.uk/~paediat/stars/). Allele and ST assignments were made using the Multi Locus Sequence Typing Web site (available at: http://spneumoniae.mlst.net). All alleles not already present in the pneumococcal MLST database were verified by resequencing the gene fragment on both strands. For STs found among isolates of >1 serotype, the MLST loci were resequenced, and, if necessary, serotypes were confirmed by Quellung reaction.

Statistical analysis. The goal was to determine whether the pneumococcal populations in these communities differed between the 2 time periods studied and, if so, to assess how this occurred. We first created a "population snapshot" using e-BURST (version 3; available at: http://eburst.mlst.net), which infers and displays the overall population structure and recent relationships between STs [16]. This program groups related STs into clonal complexes (CCs), identifies the probable ancestor of each CC as the ST with the largest number of single locus differences, and outputs a graphical representation of these relationships. This enabled us to assess the relative frequency of different STs and CCs of related strains in different samples. To assess whether the difference in composition of the pneumococcal populations from the 2 time periods was statistically significant, we used a permutation test based on a classification index, as described elsewhere [17]. Finally, differences in the diversity of STs were assessed using Simpson's D [18], with 95% confidence intervals estimated using the method of Grundmann et al. [19]. The frequencies of STs within individual serotypes were compared using Fisher's exact test. SAS statistical software was used in the analysis (version 9; SAS Institute).

RESULTS

Distinct pneumococcal populations in 2001 and 2004. We observed substantial changes in the pneumococcal populations in these communities between 2001 and 2004. The 2001 sample of 126 strains contained 61 distinct STs expressing 23 capsular serotypes. Eighteen STs were new to the MLST database. The 2004 sample of 222 strains contained 85 distinct STs expressing 30 capsular types. Of these, 29 STs were new to the database (in addition to those in the 2001 data set). Of the 119 distinct STs in the combined data sets, only 27 were found in both. Full details of strains used in this work, including allelic profiles, serotypes, penicillin susceptibility according to CLSI breakpoints, and the communities from which they were retrieved, are shown in tables 1 and 2. Significant differences in the carried pneumococcal population were found between the 2 time periods studied (classification index, D statistic = .443; $P < .01$ [17]), although no significant change was found in the overall diversity of STs composing the sample as measured by Simpson's D [18].

Changes in CCs revealed by eBURST analysis. The relationships among all isolates studied are shown as a "population snapshot" generated by eBURST analysis in figure 1*A*. Fifty percent of isolates in 2001 and 64% of isolates in 2004 fell into the same 10 specific lineages or CCs. The results of the analysis and the serotypes found in these 10 most common CCs are shown in table 3. Figure 1*B* shows the relative frequency of each CC within each sample, together with the frequencies of PNSP in each. With the exception of group 8, all CCs and the predicted ancestral ST in each were present in both time periods.

STs responsible for expansion of specific serotypes: evidence for the success of PNSP. Serotypes 19A and 35B, together with serogroup 15, showed significant increases between the 2 time periods, and, in all, expansion favored PNSP. MLST analysis allowed us to determine specifically which STs were responsible for this expansion. Among both 19A and 15 isolates, the predominant ST in 2001 was ST 199; that is, the predicted ancestor of CC5 exists in 2 forms: one with serotype 19A (ST 199[19A]) and the other with a serotype 15B/C capsule. Of interest, ST 199[19A] is associated with PNSP, whereas ST 199[15B/C] is not.

Between 2001 and 2004, the proportion of ST 199 isolates in both 19A and 15 decreased, but through different mechanisms: for the 19A serotype, several STs expanded, with the exception of ST 199. Specifically, 9 STs that were not found in 2001 were present in 2004, all of which (with the exception of a single isolate of ST 415) were PNSP. eBURST analysis allowed us to further characterize how these 19A strains are related. The 2 STs found among 19A strains in 2001 (199 and 1936) were single locus variants of each other and were, hence, considered to be part of the same CC. Of the 9 additional 19A STs that were found in 2004, only 3 fell into the same CC as ST 199. The remaining 6 STs (63, 276, 320, 415, 1925, and 1944) were all distantly related to each other (none had >2/7 alleles in common). Hence, the expansion of 19A occurred through multiple, distantly related strains.

In contrast, the expansion of serogroup 15 isolates resulted from a single serotype 15A clone (ST 63) and close relatives forming CC8. This clone was also associated with PNSP (11/12 strains). It corresponded to the PMEN Sweden 15A-25, previously described in Sweden, Spain, and Portugal [14], and showed the single largest percentage increase of any ST.

Serotype 35B strains offer a further example of clonal expansion. In this serotype, which also increased significantly between 2001 and 2004, the increase was almost entirely due to ST 558 (CC10). Again, this ST has been previously recorded as a penicillin-resistant clone with a 35B capsule in the MLST database.

Thus, we document 2 contrasting modes of serotype expansion: 1 serotype (19A) increased through the expansion or introduction of multiple rare STs, whereas others (15A and 35B) increased through a single ST. In all cases, these expansions favored PNSP.

Multiple serotypes within single STs: capsular switching? The association of a single ST or CC with >1 serotype indicates a history of capsular switching (table 3). By comparison with the MLST database, we were able to determine whether these ST/serotype associations were previously known. The serotypically mixed STs 63, 199, and 320 have been previously associated with all the serotypes reported here [11]. However, ST 156, the Spain 9V-3 multiresistant clone, was, in the present study, most commonly associated with a 9A capsule—a combination that has not previously been noted. Futhermore, 2 strains with ST 1390, which has been mainly found in 6A strains, were found to aggregate with pool I serum, indicating a capsule of type 25, 38, 43, 44, 45, 46, or 48. No further delineation of the capsule type was possible with type-specific serum. Importantly, we found no examples of successful PNSP clones in the postvaccine sample that could have arisen through capsular switching.
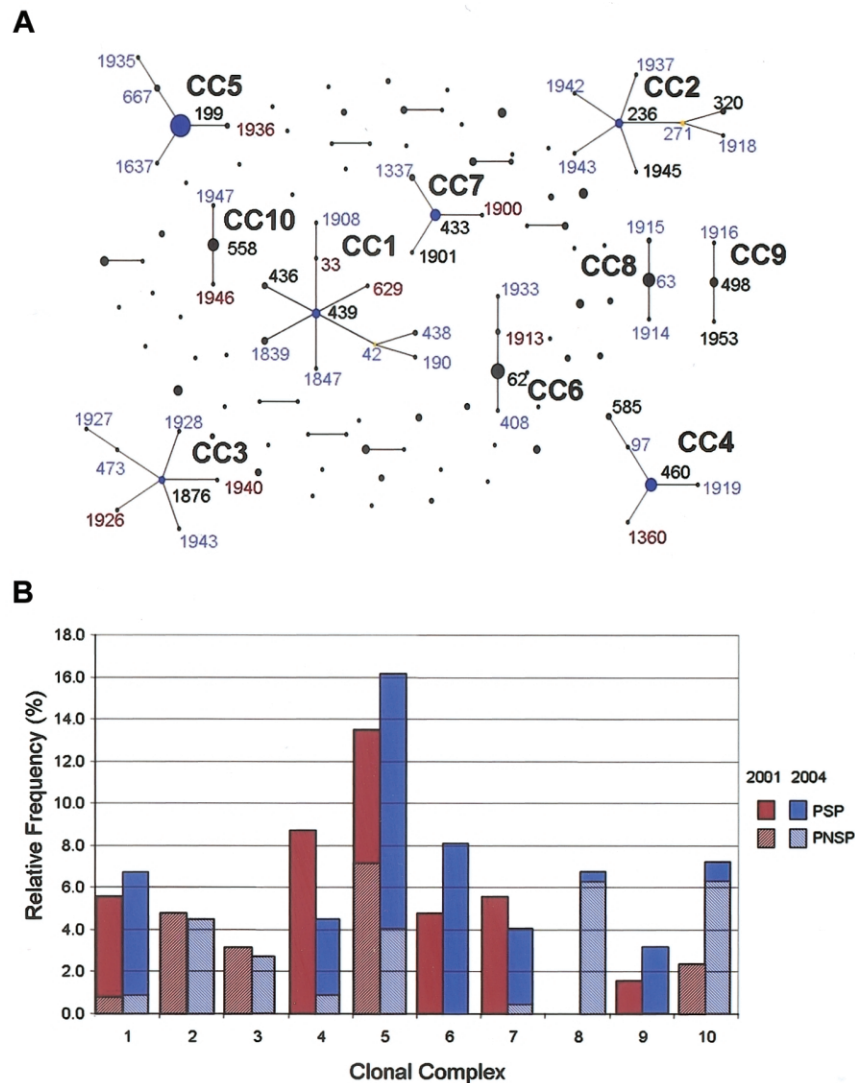
Figure 1. *A,* Population snapshot of the entire data set of 348 sequence types (STs) in 2001 and 2004. Each ST is represented by a point, the size of which is determined by the no. of isolates with that ST in the data set. STs differing at a single genetic locus are shown linked by a line. A clonal complex (CC) is a group of STs sharing 6 of 7 alleles with at least 1 other member of the group. More information is available at: http:// spneumoniae.mlst.net/eburst/. The 10 most frequent CCs in the combined data set are indicated in bold. For clarity, only the nos. of those STs in these CCs are shown. Those in black were found in both samples, those in red were found only in 2001, and those in blue were found only in 2004. *B,* Bar chart showing the relative frequency of STs within the 10 most common CCs within each sample. The proportion of these that were penicillin nonsusceptible is shown by the striped bars. PNSP, penicillin-nonsusceptible pneumococci; PSP, penicillin-susceptible pneumococci.

DISCUSSION

PCV7 has markedly reduced invasive infections among children [20]. However, the fact that only 7 of >90 serotypes are targeted raises the question of whether NVTs will expand into the niche formerly occupied by vaccine serotypes. As previously rare serotypes become prevalent carriage strains, they may reveal a previously unsuspected potential for disease in different contexts. We have previously reported decreases in the prevalence of vaccine serotypes and increases in NVTs carried among generally healthy children in 16 Massachusetts communities between 2001 and 2004 [5]. We have now applied MLST as a

means of determining, in detail, how the carriage population of pneumococci has changed as PCV7 has been introduced. We report 2 major findings: first, although we found no evidence for serotype switching whereby major vaccine type PNSP clones converted to novel NVT strains capable of evading the vaccine, as has been feared [21], we did find clear evidence for the expansion of multiple PNSP clones among NVT strains. In all cases, these expanding strains could be traced either to the 2001 sample or to strains previously identified elsewhere in the world. This suggests that previously identified CCs are now occupying the niche vacated by PNSP vaccine strains. Second,

Table 3. Details of the 10 most-common clonal complexes (CCs) as defined by eBURST in the combined data set of 348 isolates.

CC (putative ancestor)	CCs, no. (%) 2001 (n = 126)	CCs, no. (%) 2004 (n = 222)	STs, no.	PNSP strains, no. 2001	PNSP strains, no. 2004	Serotypes (no.) 2001	Serotypes (no.) 2004
1 (ST 439)	7 (5.6)	15 (6.8)	10	1	2	23A (2), 23B (2), 23F (3)	23A (8), 23B (6), 23F (1)
2 (ST 236)	6 (4.8)	10 (4.5)	8	6	10	19F (5), 9A (1)	19F (10), 19A (1)
3 (ST 1876)	4 (3.2)	6 (2.7)	7	4	6	6A (4)	6A (6)
4 (ST 460)	11 (8.7)	10 (4.5)	5	0	2	6A (10), 10 (1)	6A (7), 10 (3)
5 (ST 199)	17 (13.5)	36 (16.2)	5	9	9	15B/C (7), 19A (9), 19F (1)	15B/C (19), 19A (15), 19F (1)
6 (ST 62)	6 (4.8)	18 (8.1)	4	0	0	11A (6)	11A (18)
7 (ST 433)	7 (5.6)	9 (4.1)	4	0	1	22F (7)	22F (9)
8 (ST 63)	0 (0)	15 (6.8)	3	0	14	…	15A (14), 19A (1)
9 (ST 498)	2 (1.6)	7 (3.2)	3	0	0	35F (2)	35F (7)
10 (ST 558)	3 (2.4)	16 (7.2)	3	3	14	35B (3)	35B (15), NT (1)
Others	63 (50.0)	80 (36.0)		19	18		
Total				42	76		

NOTE. CCs are ranked by the no. of sequence types (STs) over both time periods. PNSP, penicillin-nonsusceptible pneumococci.

the increase in PNSP among NVTs may be due to a broad expansion of unrelated clones (19A) or to the expansion of a single CC (15A and 35B). The expansion of these serotypes, resulting in a significant increase in 2004, may have been driven by the selective advantage of PNSP strains in a population in which antibiotic use is very common.

The different mechanisms by which serotypes 19A, 15A, and 35B have expanded may be readily explained by the preexisting populations of PNSP within these serotypes. Several 19A PNSP clones have been reported from other locales, some of which have contributed to the change in the population described here, whereas only a single 15A serotype PNSP clone has been previously known—ST 63. Similarly, the serotype 35B clone ST 558 has been previously reported to be penicillin resistant and was present in Massachusetts before the widespread use of PCV7 (in the 2001 sample), so this appears to be another case of selection for preexisting resistance. Although it appears to be important, penicillin nonsusceptibility is not essential for success, as we observed from ST 62, a fully susceptible 11A clone that increased from 3% in 2001 to 7% in 2004. We, therefore, suggest that if NVT strains exhibit variation in antibiotic susceptibility, less-susceptible strains will have a selective advantage in vaccinated populations. In time, we would expect the more-common fully susceptible 11A strains (as well as other serotypes) to acquire resistance, after which we would predict expansion of resistant strains. However, whether this will be the case can only be determined by further surveillance.

MLST allows us to distinguish between nonvaccine PNSP arising through serotype switching, through de novo acquisition, or through expansion of previously recognized strains. Although others have documented capsular switching by particular clones (such as the 9V-Spanish clone to an 11A serotype [22]), we found no examples of capsular switching involving highly resistant clones of vaccine serotypes over the sampling periods studied here. However, we predict that such events would be more likely in communities in which PNSP clones predominate and are, therefore, likely to be involved in such genetic exchange in the future.

In terms of limitations, the present study uses a baseline sample taken before full implementation of vaccine, rather than a true prevaccination sample. Thus, the effects due to vaccination may be underestimated. However, although we document changes in the population, we are unable to definitively ascribe those changes to specific causes—namely, vaccination or antibiotic use. Nevertheless, we are unaware of any features of the studied populations other than vaccination that are likely to explain the data we present.

A critical remaining issue is the extent to which colonizing NVTs will become increasingly responsible for disease in humans—both invasive (e.g., bloodstream and central nervous system infections) and localized (e.g., otitis media and sinusitis). Although the relationship is complex, nasopharyngeal colonization is a precursor to IPD [9]. The increase in the fraction [23] and even the absolute number [24] of IPD cases caused by NVT isolates is a cause for concern, although the overall incidence of IPD (and the prevalence of resistance among invasive infections) is much reduced, compared with that observed before PCV7 introduction [25]. However, serotype replacement among certain vulnerable patient groups, such as those who are immunosuppressed for various reasons, is recognized as a growing problem. As for local infections, there is little variation in the ability of colonizing serotypes to cause acute otitis media (AOM) [26]. Hence, increasing colonization with antibiotic-nonsusceptible NVTs is likely to increase their roles as pathogens in AOM. Therefore, continued assessment of the incidence of both IPD and AOM (and other localized

infections) as colonizing serotypes change will be necessary to understand the long-term impact of PCV7 immunization.

Using MLST to study pneumococcal carriage under increasing selective pressure from conjugate vaccination, we have demonstrated significant changes in the population and defined the strains and CCs responsible. Although serial sampling in defined communities is a strength of this work, surveillance from other regions will be necessary to assert the generalizability of these findings. With these caveats, these data suggest that vaccine introduction, together with the selective pressure of antibiotic use, will continue to lead to increasing frequency of PNSP among NVTs. Changes in the pneumococcal population are unlikely to substantially abrogate the benefits of vaccination in the near future, but the significant association of 19A with IPD [27] is cause for concern, especially because this serotype was found to contain multiple, unrelated PNSP lineages. Furthermore, even if pneumococcal strains replacing those formerly prevalent in the population are less invasive than the previously carried strains, they may be increasingly responsible for localized infections such as AOM or sinusitis. Continuing careful surveillance of the carried pneumococcal population and prevalence of antibiotic-resistant strains is essential to guide treatment decisions and inform future vaccine development.

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