MAJOR ARTICLE

Postvaccine Genetic Structure of *Streptococcus pneumoniae* Serotype 19A from Children in the United States

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Background. The introduction of the 7-valent conjugate pneumococcal vaccine (PCV7) in children may result in serotype replacement. We estimated the rate of increase of invasive pneumococcal disease (IPD) caused by serotype 19A in children <5 years old and determined the genetic composition of these isolates.

Methods. Cases of IPD between July 1999 and June 2004 were identifie through the Active Bacterial Core Surveillance. Serotype 19A isolates obtained from children <5 years old between January 2003 and June 2004 were characterized by serotyping, antibiotic susceptibility testing, and pulsed-fiel gel electrophoresis (PFGE). Select isolates representing homologous PFGE clusters were subjected to multilocus sequence typing, and eBURST was used to delineate clonal groups.

Results. Between July 1999 and June 2004, the overall rate of IPD decreased from 23.3 to 13.1 cases/100,000 population ($P < .00001$). In children <5 years old, the rate decreased from 88.7 to 22.4 cases/100,000 population ($P < .00001$), whereas the rate in persons ⩾5 years old decreased from 18.4 to 12.4 cases/100,000 population ($P < .0001$). The rate of serotype 19A IPD in children <5 years old increased significantl from 2.6 cases/100,000 population in 1999–2000 to 6.5 cases/100,000 population in 2003–2004; this was accompanied by significan increases in penicillin nonsusceptibility ($P = .008$) and multidrug resistance ($P = .002$) among serotype 19A isolates. As was observed during the pre-PCV7 era, clonal complex (CC) 199 predominated within serotype 19A, representing ~70% of invasive serotype 19A isolates from children <5 years old during 2003–2004. New serotype 19A genotypes were observed during 2003–2004, including 6 CCs that were not found among pneumococcal serotype 19A isolates during surveillance in 1999.

Conclusion. Serotype 19A is, at present, the most important cause of IPD by replacement serotypes, and it is increasingly drug resistant. CC199 is the predominant CC among type 19A serotypes in children <5 years old. Our data suggest that some of the increase in rates of infection with serotype 19A may be due to serotype switching within certain vaccine type strains.

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide, particularly in young children, elderly persons, and immunocompromised individuals of all ages. Pneumococci are responsible for a wide variety of disease manifestations, ranging from mild upper-respiratory-tract illness to more severe, life-threatening infections that include bacteremia and meningitis. Globally, pneumococci cause ~800,000 deaths annually due to pneumonia and meningitis in young children, mostly in developing countries [1]. In the United States alone in 2000, *S. pneumoniae* caused an estimated 17,000 cases of invasive disease in children <5 years old, including 700 cases of meningitis [2]. However, the development of conjugate vaccines holds great promise for reducing the burden of invasive pneumococcal disease (IPD) in young children [3].

The 7-valent pneumococcal conjugate vaccine (PCV7; Prevnar) was licensed for use in the United States in 2000 for young children, and it is recommended for all children <2 years old and children 2–4 years old who have certain chronic illnesses [2]. Since the introduction

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of the vaccine, a significan decrease in rates of IPD caused by the serotypes covered by the vaccine has been observed [4]. However, little is known about the long-term impact of the vaccine, and the extent to which vaccine serotypes (VTs) will be replaced by nonvaccine types (NVTs) is still not clear. Although the replacement of VTs by NVTs has been demonstrated in vaccinated children in studies of nasopharyngeal colonization and of otitis media [5, 6], the picture remains less clear for invasive strains. Early reports have suggested some increase in numbers of NVTs among invasive isolates [4, 7]. However, none of the studies performed since the introduction of the vaccine have investigated the individual clones within the replacing serotypes to document the number of genetically divergent lineages, the appearance of new clones, and/or the expansion of existing clones. The relative importance of serotype and genotype in determining the invasive potential of the organism is still debated. A meta-analysis that took into consideration the relative prevalence of individual clones and serotypes among isolates from patients with IPD, compared with their prevalence in carriage isolates, suggested that the serotype is the most important determinant for causing serious infections [8]. Other studies have clearly demonstrated differences in the potential of different clones within the same serotype to cause IPD [9, 10]. As these issues continue to be discussed, a detailed characterization of the genetic background of isolates obtained during the postvaccine period may provide invaluable insights concerning the clonal composition of NVTs involved in replacement.

The Active Bacterial Core Surveillance (ABCS), part of the Centers for Disease Control and Prevention's Emerging Infections Program, has conducted active, population-based, laboratory-based surveillance for IPD since 1995. After the introduction of PCV7, an apparent increase in the proportion of cases of IPD caused by serotype 19A in children <5 years old was observed. The present study was undertaken to determine whether this change represented a true increase in the incidence of serotype 19A disease in children <5 years old and to obtain more insights into the potential impact of the vaccine on the genetic composition of invasive serotype 19A isolates. We report a significan increase in the incidence of serotype 19A disease in children <5 years old, with isolates of multilocus sequence type (ST) 199 and related STs representing the predominant clonal complex (CC). Additionally, we detected several genotypes among serotype 19A that have been previously associated with serotypes targeted by PCV7.

PATIENTS, MATERIALS, AND METHODS

Patients. ABCS has monitored invasive pneumococcal infections since January 1995 [11]. Cases of IPD were define by the isolation of pneumococci from a normally sterile site in residents of the surveillance population, as described elsewhere [12]. The analysis of trends over time in the incidence of IPD caused by serotype 19A was limited to areas under continuous surveillance from July 1999 until June 2004 [12]. These areas included California (San Francisco County), Connecticut, Georgia (20 counties in the Atlanta area), Maryland (6 counties in the Baltimore area), Minnesota (7 counties in the Twin Cities area), New York (7 counties in the Rochester area and 8 counties in the Albany area), Oregon (3 counties in the Portland area), and Tennessee (4 urban counties). The total population in 2004 for these surveillance areas was 19,043,870 persons, according to US Census data, with a population of 1,269,793 children <5 years old.

Isolates. One hundred twenty-seven isolates of serotype 19A obtained during January 2003–June 2004 from children <5 years old were included in the study. The 4 internationally dispersed clones of serotype 19A (Hungary^{19A}-6, South Africa^{19A}-7, Czech Republic^{19A}-11, and South Africa^{19A}-13) included for comparison were obtained from the Pneumococcal Molecular Epidemiology Network (PMEN; http://www.sph.emory.edu/PMEN).

Serotyping. Isolates were serotyped by latex agglutination and confi med by Quellung reaction.

Antimicrobial susceptibility testing. MICs were determined by the microbroth dilution method. The antimicrobials tested included penicillin, cefotaxime, choramphenicol, tetracycline, clindamycin, erythromycin, rifampin, levofloxacin vancomycin, and trimethoprim-sulfamethoxazole (TMP-SMZ). MICs were interpreted using NCCLS guidelines [13]. Intermediate and resistant isolates were grouped together as being nonsusceptible. Multiple drug resistance was define as isolates that were nonsusceptible to ⩾3 of the following antibiotics: penicillin, erythromycin, TMP-SMZ, tetracycline, chloramphenicol, clindamycin, rifampin, and levofloxacin

Pulsed-fiel gel electrophoresis (PFGE) and multilocus sequence typing (MLST). PFGE was performed as described elsewhere [14]. The bacterial plugs were treated with 30 U of *Sma*I and loaded onto a 1% gel, and PFGE was performed again as described elsewhere [15]. MLST was performed as described elsewhere [14, 16]. The sequence types were determined using the Wisconsin package (version 10.3; Accelrys) with alleles downloaded from the Multi Locus Sequence Typing Home Page (available at: http://www.mlst.net).

PFGE analysis. The unweighted pair-group method with arithmetic averages (UPGMA) method was used to obtain dendrograms of banding patterns with the dice coefficient Bionumerics software (version 2.5; Applied Maths) was used for analysis as described elsewhere [14]. Isolates with ⩾80% identity were considered to represent the same PFGE cluster. PFGE was used as a screen to identify clusters to be characterized further by MLST, because our previous studies have shown that isolates within the same serotype that share similar PFGE patterns with UPGMA dice coefficient of ⩾80% are almost always found to share ⩾5 identical alleles by MLST [14].

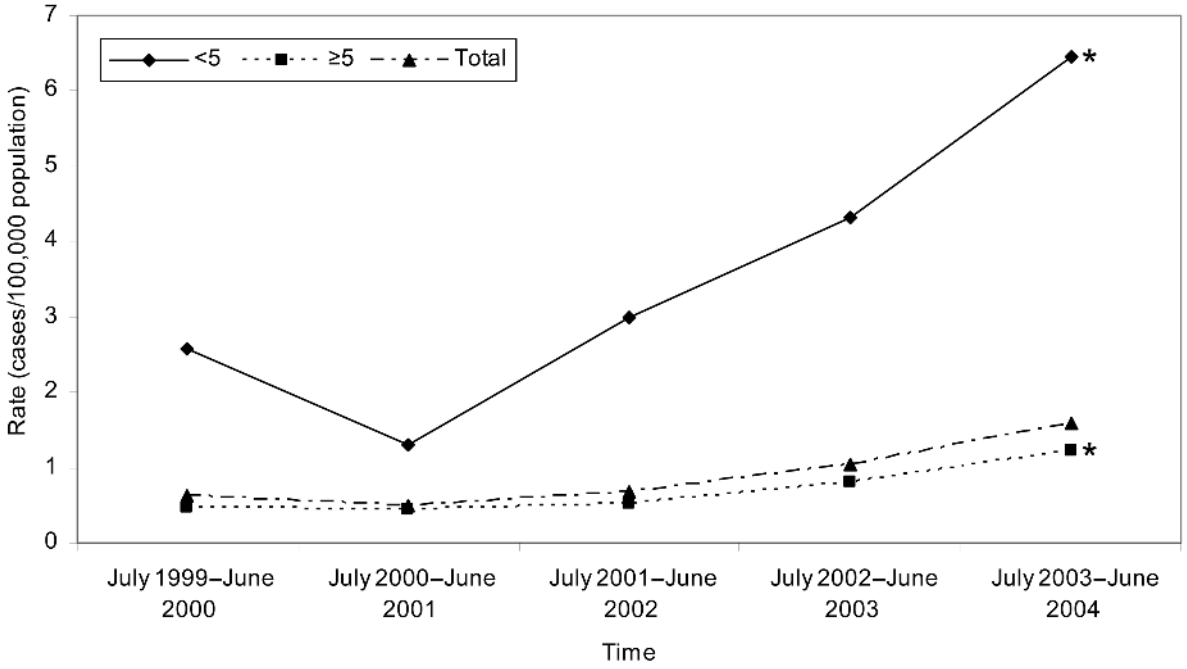

Figure 1. Projected incidence rates of invasive pneumococcal infections caused by serotype 19A, Active Bacterial Core Surveillance sites, July 1999–June 2004. Asterisks indicate a 2.5-fold increase in rate in children <5 years old, July 2003–June 2004 vs. July 1999–June 2000 ($P < .00001$), and a 2.6-fold increase in rate in persons ⩾5 years old, July 2003–June 2004 vs. July 1999–June 2000 ($P < .00001$).

MLST analysis. New MLST profile obtained during the present study were submitted to the curator at http://spneumoniae.mlst.net for designations. CCs were assigned using the eBURST algorithm [17], with the software available from the Multi Locus Sequence Typing Home Page. A clonal group (CG) was define as one in which all STs are single locus variants (SLVs) of at least 1 other ST within the group. The founder ST was define as the ST within the CG with the greatest number of SLVs and was determined on the basis of the data at the global database, even when a founder type was not seen in our study set. A CC, for the present work, consisted of CGs, or CGs plus 1 related ST, that shared 5 of 7 allelic identities with at least 1 ST within the CG, or, in 1 case, a founder ST plus a single triple-locus variant.

Statistical analysis. Data were analyzed with SAS (version 9.1; SAS Institute) and StatXact (version 6.2.0; Cytel Software) software. Proportions were compared using the χ^2 test, Fisher's exact test, or Cochrane-Armitage test for trend, as appropriate. $P < .05$ was considered to be significant Cumulative annual incidence rates were calculated from July 1999 through June 2004 using population estimates from the US Census Bureau for each calendar year. For example, the estimated midyear population for July 2003 was used as the denominator for all cases occurring between 1 July 2003 and 30 June 2004. To calculate serotype-specifi incidence rates, case patients with missing serotype information were redistributed by age and race under the assumption that the frequency was the same as that for case patients with serotype information.

RESULTS

Between July 1999 and June 2004, 15,944 cases of IPD occurred in the surveillance areas. During this same period, the overall rate of IPD decreased from 23.3 to 13.1 cases/100,000 population ($P < .00001$). In children <5 years old, the rate decreased from 88.7 to 22.4 cases/100,000 population ($P < .00001$), whereas the rate in persons ⩾5 years old decreased from 18.4 to 12.4 cases/100,000 population ($P < .0001$). Serotype information was available for 14,198 (89%) cases. Of these, 759 (5.3%) were known to be serotype 19A. After unknown serotypes were proportionately allocated, the estimated number of cases of serotype 19A disease that occurred in children <5 years old was 33 in 1999–2000, 17 in 2000–2001, 39 in 2001–2002, 57 in 2002–2003, and 86 in 2003–2004. The estimated incidence rate of serotype 19A IPD in children <5 years old increased from 2.6 cases/100,000 population in 1999–2000 to 6.5 cases/100,000 population in 2003–2004 (rate ratio [RR], 2.5 [95% confidenc interval {CI}, 1.7–3.7]) (figu e 1). In persons ⩾5 years old, the rate of serotype 19A disease increased from 0.48 to 1.2 cases/100,000 population (RR, 2.6 [95% CI, 2.0–3.4]). Because the absolute increase in incidence was greatest in children <5 years old, compared with persons ⩾5 years old, and because PCV7

is only recommended for children <5 years old, we focused the remainder of our analysis on this age group. A significan increase ($P = .008$) in penicillin nonsusceptibility among serotype 19A isolates was also observed that was concurrent with the increase in the rate of serotype 19A IPD in children <5 years old (figu e 2). Finally, the proportion of strains with full penicillin resistance and multidrug resistance also increased significantl ($P = .001$ and $P = .002$, respectively). Nonsusceptibility to TMP-SMZ (56%) and erythromycin (38%) among serotype 19A isolates obtained during 2003–2004 was also high. All isolates were susceptible to vancomycin and levofloxacin

A total of 127 isolates were collected from children <5 years old during January 2003–June 2004; these included isolates from each surveillance site. The majority of these isolates were recovered from children with bacteremia (without an apparent focus, 45%; bacteremic pneumonia, 43%); 4% were from children with meningitis.

Clonal analysis by PFGE and MLST. PFGE analysis of the 127 isolates showed 84 (66%) isolates that shared a single conserved cluster and several additional diverging clusters (figu e 3). One distinct cluster of 17 isolates (13%) was the only other large cluster. Only 1 isolate in the study showed >80% similarity to Czech Republic[19A]-11, whereas the other PMEN clones appeared to be distinct from the study isolates. The largest cluster primarily consisted of penicillin-susceptible isolates (36/84 [43%]) and penicillin-nonsusceptible isolates with intermediate resistance (44/84 [52%]), whereas only 4 isolates (5%) were fully resistant to penicillin. Twenty-six percent ($n = 22$) of the isolates in this cluster were also multidrug resistant (figu e 3). However, a greater number ($n = 29$) of isolates with multidrug resistance were represented on the lower end of the dendrogram, with banding patterns that differed distinctly from the major PFGE cluster (figu e 3).

MLST was used to accurately group isolates into CCs and compare them with our prevaccine data. The strains in the study were grouped into 8 CCs (figu e 4), with CC199 being the largest CC corresponding to the large PFGE cluster (figu e 3). Five additional isolates sharing ~75% PFGE pattern similarity with the large cluster were grouped into ST199 and ST1341 (the double-locus variant [DLV] of ST199). ST199 was the predicted founder of this CC when all known STs were used, and it was the predominant allelic profil listed among

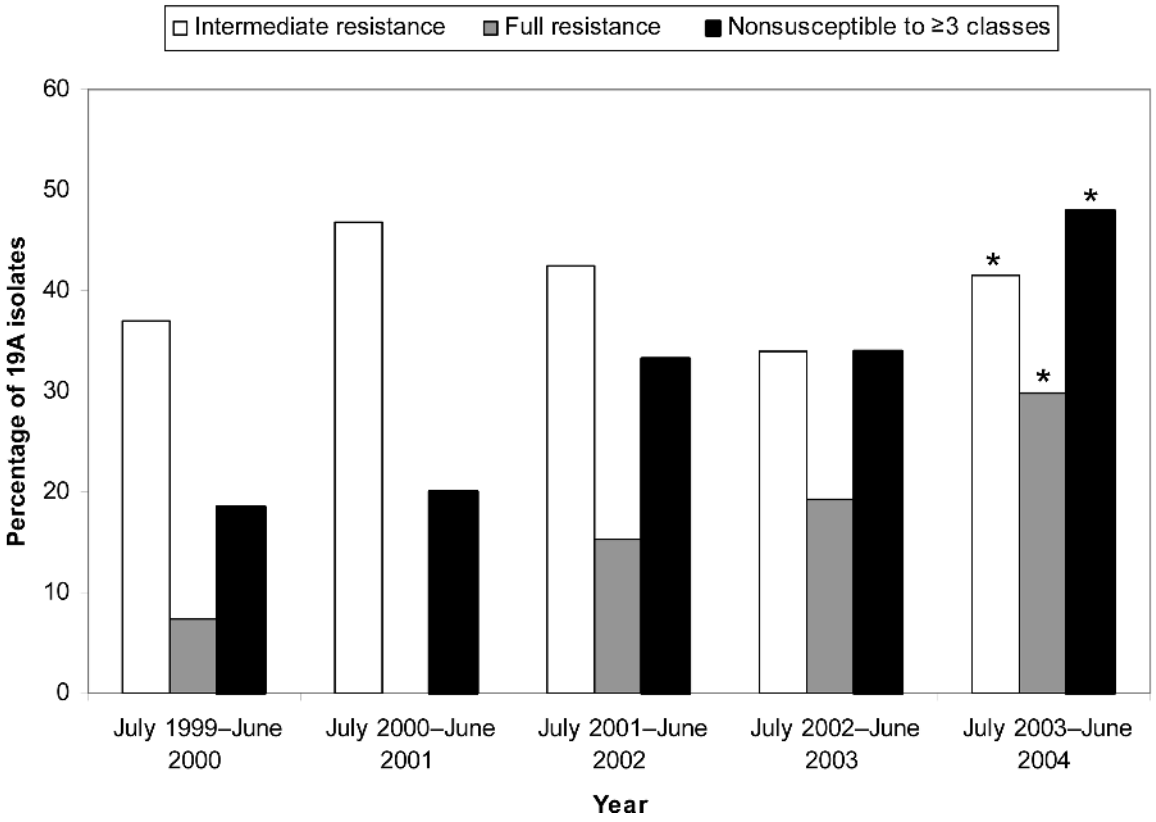

Figure 2. Proportion of all invasive pneumococcal isolates of serotype 19A with intermediate or full penicillin resistance and nonsusceptibility to ⩾3 antibiotic classes in children <5 years old, Active Bacterial Core Surveillance sites, July 1999–June 2004. Asterisks indicate a 61% increase in the percentage of penicillin-nonsusceptible (intermediate and resistant) strains ($P = .008$), a 303% increase in the percentage of penicillin-resistant strains ($P = .001$), and a 159% increase in the percentage of strains nonsusceptible to ⩾3 classes of antibiotics ($P = .002$). All P values were computed using the Cochrane-Armitage trend test.

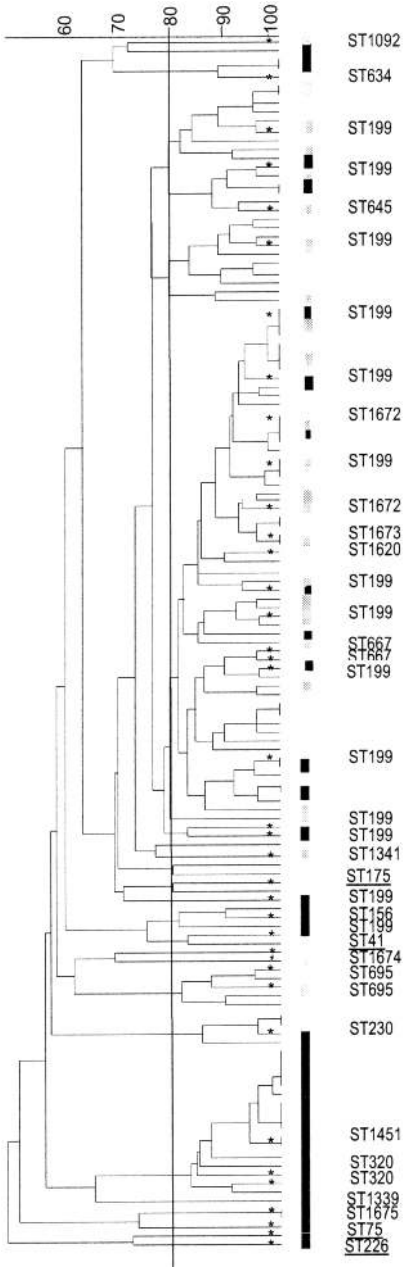

Figure 3. Genetic composition of serotype 19A isolates from children with invasive disease. A pulsed-field gel electrophoresis dendrogram (unweighted pair-group method with arithmetic means) of 127 serotype 19A Active Bacterial Core Surveillance isolates collected during 2003–2004 is shown. Dice coefficients are shown above the dendrogram. Isolates with ⩾80% relatedness on the dendrogram were considered to be highly genetically related (a line indicating the 80% marker runs the length of the dendrogram). *Vertical gray lines,* isolates with intermediate resistance to penicillin; *vertical black lines,* isolates with multidrug resistance. Multilocus sequence type (ST) results are correlated to specific isolates depicted in the dendrogram. Asterisks indicate isolates subjected to multilocus sequence typing. Multilocus STs corresponding to Pneumococcal Molecular Epidemiology Network clones are underlined.

serotype 19A isolates described in the extensive global database at http://spneumoniae.mlst.net. Through the course of the study, 5 new STs (ST1620, ST1672, ST1673, ST1674, and ST1675) were discovered, and 4 of these were SLVs of ST199, which expanded the number of STs included within the CC.

The other large cluster of isolates ($n = 17$) as seen by PFGE analysis was represented by CC271. The STs of this large CC (>70 STs listed in global databases) have been reported primarily among type 19F strains, including the internationally disseminated multidrug-resistant clone Taiwan^{19F}-14 (ST236; see http://www.sph.emory.edu/PMEN/). CC271 isolates in the present study were represented by the SLVs ST1451 and ST320 (figu es 3 and 4). ST1451 is a DLV of both the founder ST271 and of ST236, whereas ST320 is an SLV of ST271 and a DLV of ST236. Our CC271 isolates were fully resistant to penicillin and cefotaxime and accounted for ~35% of the multidrug-resistant strains in the study. CC271 isolates were not observed among serotype 19A isolates from 1999 [14].

The other STs observed among serotype 19A, accounting for 16% of the 127 isolates, were within the 6 CCs CC247 (ST695), CC230 (ST230), CC1296 (ST1339 and ST1675), CC490 (ST1092), CC156 (ST156), and CC81 (ST634) (figu es 3 and 4), 4 of which were composed of multidrug-resistant isolates. Thus, the 6 CCs CC247, CC230, CC1296, CC490, CC156, and CC271 were observed among ABCS serotype 19A isolates from 2003–2004, but they were not observed among pneumococcal isolates from 1999 (figu e 4). ST695 (which is primarily associated with serotype 4), ST230 (which is primarily associated with 14 and 24F), ST1092 (which is primarily associated with serogroup 6), and ST156 (which is primarily seen within serotype 9V [14]) have not been previously reported in the global database among serotype 19A strains, and they appear to be new associations that may have occurred as a result of capsular switching.

The appearance of ST156 among multidrug-resistant serotype 19A isolates is reason for concern, because ST156 indicates the internationally prevalent Spain^{9V}-3 clone. Also interesting was the appearance of CC1296 among the serotype 19A isolates characterized. This CC, which is represented by the multidrug-resistant international clone North Carolina^{6A}-23 (ST376, a DLV of both ST1296 and ST1339), was highly prevalent among type 6A isolates in 1999 [14]. The appearance of ST695 among 5 genetically related isolates of serotype 19A is also troubling, because this sequence type has been seen exclusively among serotype 4 isolates [14], an important pediatric serotype before the introduction of PCV7. Four of these 5 isolates were susceptible to all the antimicrobials tested, and the fift was intermediately resistant to penicillin.

DISCUSSION

IPD continues to be an important cause of morbidity and mortality worldwide. The introduction of PCV7 has significantl re-

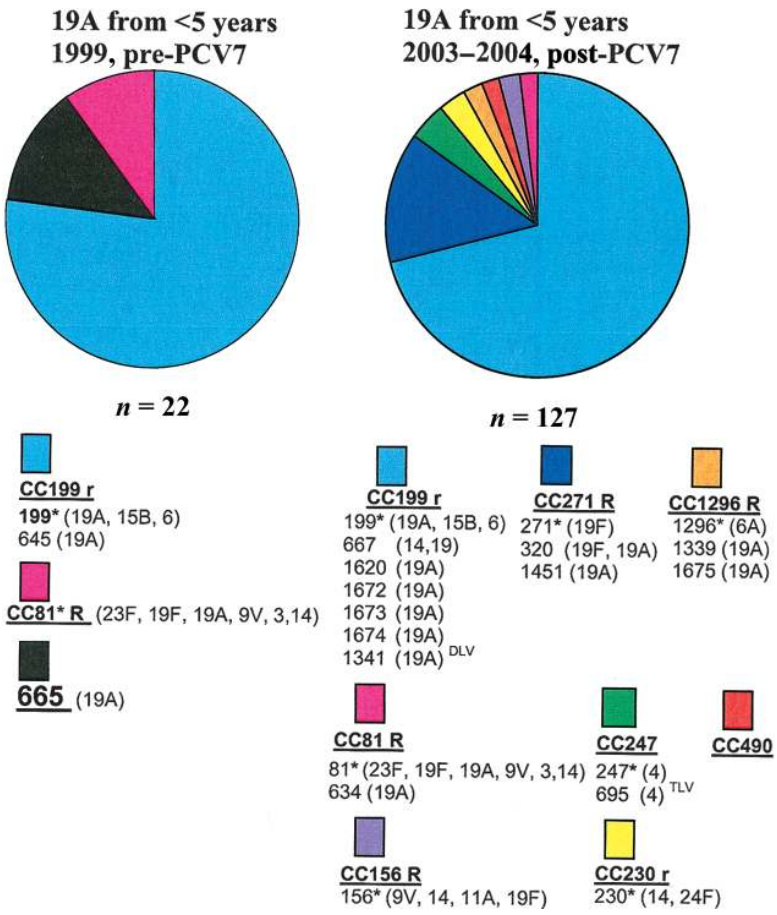

Figure 4. Clonal relationships among the 127 isolates of serotype 19A. Each clonal complex (CC) is represented in a different color and is underlined. The identification of founder members described for each CC was based on global data available at http://spneumoniae.mlst.net; these members are represented with an asterisk; serotypes in brackets indicate all serotype associations for each sequence type (ST) reported at the Multi Locus Sequence Typing Home Page. r, CCs with intermediate resistance to penicillin, often with resistance to at least 1 other class of antimicrobial; R, CCs associated with full penicillin resistance and multidrug resistance.

duced the rate of IPD in children [4] and has focused considerable attention on the postvaccine seroepidemiologic aspects of pneumococci [5, 18, 19]. Reports of the emergence of penicillin-nonsusceptible clones of NVT sharing genetic relatedness with internationally established clones targeted by the vaccine [18, 20] have indicated the need to track phenotypic and genotypic changes within invasive pneumococci. The present study, which was initiated because of our concern about the increase in the rate of IPD caused by serotype 19A pneumococci in children, has documented the genetic structure of serotype 19A isolates recovered from this vaccine target population. Our results indicate that the major pre-PCV7 CC (CC199) has expanded and reveal the introduction of serotype 19A into successful clones formerly targeted by PCV7.

We have reported an absolute increase in the rate of IPD caused by serotype 19A during the postvaccine period; the increase was largest in children <5 years old. Although no significan replacement was reported soon after the introduction of PCV7 [4], the increase in the prevalence of serotype 19A reported in the present article was seen 3–4 years after the introduction of PCV7, which could be an indicator that replacement is a gradual but steady process. Although it is difficul to attribute this increase to any single factor, reports of the efficac of the vaccine may provide some indications. The assessment of PCV7 to establish serological correlates of protection for otitis media showed that the predicted efficac for 19F was negligible even up to the highest geometric mean concentration tested [21]. Also, the measured vaccine efficac against otitis media has been found to be lowest for serotype type 19F, with the 19F antigen probably providing less cross-protection for disease caused by serotype 19A [6]. Although these factors are likely to have played major roles in the increase in the prevalence of serotype 19A strains within the niche created by decreasing vaccine types, the potential ability of specifi clones within a serotype to be biologically successful and have a higher disease potential cannot be ignored.

Genetic analysis of serotype 19A isolates showed CC199 to

be the predominant CC, accounting for 72% of the isolates in the study. This CC accounted for 77% of serotype 19A isolates before the introduction of PCV7 [14], and CC199 remains the predominant CC within serotype 19A. Several other studies have also documented ST199 as being the most important ST among clinical isolates of serotype 19A [9, 22, 23], although in smaller numbers. In a meta-analysis that compared the prevalence of individual clones (multilocus STs) and serotypes among isolates causing IPD with that of carriage strains, ST199 was found to be somewhat less invasive [8]. However, only 4 isolates of this ST were included, and 3 of them were from subjects with IPD. The ability of the isolates within this CC to expand and cause serious disease, as shown by the results of the present study, provides compelling evidence about the invasive potential of this clone.

It is also important to note that ST199 is the predominant genotype observed among serotype 15B/C infections (these 2 serotypes interconvert [24]), both in our data set and elsewhere [22, 23, 25], and it accounted for all type 15B/C isolates analyzed from children <5 years old before the introduction of PCV7 [14]. More recently, serogroup 15 has been reported to be the most frequent NVT colonizing vaccinated children, and it is increasingly common among invasive isolates obtained from hospitalized children [5, 7]. Serotype 19A and serogroup 15 strains are becoming more prevalent during the postvaccine period. Because CC199 is the predominant CC within both these serotypes, it may be the most important CC in the United States causing IPD in children <5 years old. Our study has expanded the known extent of CC199 with the discovery of 4 new SLVs of ST199, which supports the view that, as the founding genotype increases in frequency in the population, it gradually diversifie to include several newer descendants that differ from the founder at only 1 of 7 alleles [17].

The appearance of other CCs, particularly those associated with drug resistance, is noteworthy. CC271—which includes ST320 and ST1451—appears to be associated only with serogroup 19. Although CC271 accounted for 23% of our prevaccine type 19F isolates from children <5 years old, [14], we did not detect this multidrug-resistant complex among serotype 19A isolates in the present study. The appearance of multidrug-resistant ST1451 isolates within CC271 serotype 19A isolates during 2003–2004 is interesting. This ST is currently shared by only 2 other isolates (both serotype 19A and both isolated in 2000 and 2002) in the global database (http://www.mlst.net); both are multidrug resistant and were recovered in the United States. Although this subclone appears to be unique and limited by geography, its multidrug resistance could favor its international spread (if it has not already been internationally established). However, such strains have been described to emerge and disappear within short periods of surveillance [9], and they can be tracked only through reliable molecular surveillance.

Pneumococci are naturally transformable, and capsular switching is a well-documented mechanism for the potential evasion of the host immune response [26]. ST156 originated in Spain in the 1980s within serotype 9V and then spread internationally [27]. To our knowledge, the appearance of this genotype within serotype 19A has not been reported previously, and this is troubling, because this could be representative of a successful vaccine escape mechanism used by PCV7-targeted clones. Similarly, the appearance of serotype 19A isolates within CC1296, the DLVs of which include the PMEN clone North Carolina^{6A}-23, is of concern, because the PMEN clones have a proven ability to proliferate and establish themselves. The appearance of ST695 among 5 serotype 19A isolates in the present study provides further suggestive evidence of vaccine escape through capsular switching. ST695 was the most prevalent ST among pediatric type 4 isolates in our surveillance [14] (Centers for Disease Control and Prevention, unpublished data); to date, this has been observed only in association with serotype 4, a genetically stable serotype that has not been associated with drug resistance and carriage [9, 14]. ST205 (a SLV of ST695) has been also been reported among US invasive isolates [14] and among type 4 isolates with a high invasive-disease potential (OR, 12.1) [8]. The new association of this established CC with a serotype not targeted by PCV7 and increasingly encountered in young children is troubling.

Our finding describe potentially important clonal relationships within a serotype that has been increasingly found to cause IPD during the postvaccine period. However, it is not clear whether ST199 has a selection advantage over the other clones observed within serotype 19A. Many of the minor clones reported in the present study, in contrast to CC199, were multidrug resistant and may have an advantage that could help them to proliferate and establish. With time, could these minor clones predominate within serotype 19A? Also, it is possible that CC199 itself will develop a higher proportion of multidrug resistance as it continues to cause serious invasive disease. Only continued monitoring of these genetic backgrounds will reveal answers about the dynamics of the serotype 19A population.

ACTIVE BACTERIAL CORE SURVEILLANCE TEAM

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