

Strain features and distributions in pneumococci from children with invasive disease before and after 13-valent conjugate vaccine implementation in the USA

B. J. Metcalf¹, R. E. Gertz, Jr.¹, R. A. Gladstone^{2,3}, H. Walker¹, L. K. Sherwood¹, D. Jackson¹, Z. Li¹, C. Law¹, P. A. Hawkins¹, S. Chochua¹, M. Sheth⁴, N. Rayamajhi¹, S. D. Bentley^{2,3}, L. Kim¹, C. G. Whitney¹, L. McGee¹ and B. Beall¹, on behalf of the Active Bacterial Core surveillance team

1) Centers for Disease Control and Prevention, National Center for Immunization and Respiratory Diseases, Atlanta, GA, USA, 2) Wellcome Trust Sanger Institute, Hinxton, 3) Department of Medicine, Addenbrookes Hospital, University of Cambridge, UK and 4) Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases, Atlanta, GA, USA

Abstract

The effect of second-generation pneumococcal conjugate vaccines on invasive pneumococcal disease (IPD) strain distributions have not yet been well described. We analysed IPD isolates recovered from children aged <5 years through Active Bacterial Core surveillance before (2008–2009; $n = 828$) and after (2011–2013; $n = 600$) 13-valent pneumococcal conjugate vaccine (PCV13) implementation. We employed conventional testing, PCR/electrospray ionization mass spectrometry and whole genome sequence (WGS) analysis to identify serotypes, resistance features, genotypes, and pilus types. PCV13, licensed in February 2010, effectively targeted all major 19A and 7F genotypes, and decreased antimicrobial resistance, primarily owing to removal of the 19A/ST320 complex. The strain complex contributing most to the remaining β -lactam resistance during 2011–2013 was 35B/ST558. Significant emergence of non-vaccine clonal complexes was not evident. Because of the removal of vaccine serotype strains, positivity for one or both pilus types (PI-1 and PI-2) decreased in the post-PCV13 years 2011–2013 relative to 2008–2009 (decreases of 32–55% for PI-1, and >95% for PI-2 and combined PI-1 + PI-2). β -Lactam susceptibility phenotypes correlated consistently with transpeptidase region sequence combinations of the three major penicillin-binding proteins (PBPs) determined through WGS analysis. Other major resistance features were predictable by DNA signatures from WGS analysis. Multilocus sequence data combined with PBP combinations identified progeny, serotype donors and recipient strains in serotype switch events. PCV13 decreased the frequency of all PCV13 serotype clones and concurrently decreased the frequency of strain subsets with resistance and/or adherence features conducive to successful carriage. Our results serve as a reference describing key features of current paediatric IPD strains in the USA after PCV13 implementation.

Clinical Microbiology and Infection published by Elsevier Ltd on behalf of European Society of Clinical Microbiology and Infectious Diseases.

Keywords: Antimicrobial susceptibility, clonal complexes, pneumococcal conjugate vaccines, serotype distributions, whole genome sequence

Original Submission: 26 June 2015; **Revised Submission:** 28 July 2015; **Accepted:** 29 August 2015

Editor: J.-L. Mainardi

Article published online: 10 September 2015

Corresponding author: B. Beall, 1600 Clifton Rd, NE, Atlanta, GA 30333, USA
E-mail: bbeall@cdc.gov

Introduction

Streptococcus pneumoniae can quickly adapt to selective pressures through horizontal transfer and intrachromosomal mutation events. Even so, the conjugate vaccine strategy of targeting currently predominant invasive serotypes has been highly successful [1,2] and durable [3] in decreasing the

frequency of invasive pneumococcal disease (IPD) and pneumococcal antimicrobial resistance. However, individual variants that originated through serotype-switching events substantially contributed to disease burdens in the USA in the post-seven-valent pneumococcal conjugate vaccine (PCV7) era [4–8]. Although the monitoring of pneumococcal serotypes and antimicrobial susceptibilities provides the major information for devising prevention strategies, approaches that deduce the causal chromosomal changes that lead to the emergence of individual invasive strains allow for a better biological and epidemiological understanding of pneumococcal strains. Here, we have provided a strain reference that supplies distributions and genetic features of each invasive serotype that we detected in a US surveillance system during a key 5-year period of IPD surveillance.

Materials and methods

Patients

Active Bacterial Core surveillance (ABCs) is an active, population-based and laboratory-based surveillance system that is part of the CDC Emerging Infections Program. Cases of IPD were defined by the isolation of pneumococci from a normally sterile site in residents of the surveillance areas in ten different states [1–3] (see ABCs surveillance reports for population sizes and IPD incidence at <http://www.cdc.gov/abcs/reports-findings/surv-reports.html>). Throughout the entire 2005–2013 period, the number of individuals aged <5 years in the ABCs catchment area remained approximately 2.1 million.

Isolates

For the years of the primary focus (2008–2009 and 2011–2013), isolates corresponding to 84.7–90.4% of reported cases were available to the CDC *Streptococcus* Laboratory for characterization (average, 87.9%). For each of these 5 years, all of these isolates were characterized with respect to serotypes, multilocus sequence type (MLST)-based clonal complexes (CCs), resistance features, and pilin subunit presence or absence.

Isolates were characterized by use of a combination of conventional testing, PCR/electrospray ionization mass spectrometry (ESI-MS), and short-read whole genome sequence (WGS) analysis. In total, 1428 isolates, corresponding to 87.9% of the 1625 documented IPD cases in children aged <5 years, were available for characterization (828/948 for 2008–2009, and 600/677 for 2011–2013) (Supplementary Fig. S1).

Capsular serotyping

All isolates from 2005 through 2013 were serotyped with latex agglutination and the Quellung reaction employing CDC antisera.

WGS analysis

Of the 801 combined year 2009, 2012 and 2013 IPD isolates described in this study, serotypes, antimicrobial susceptibilities, MLSTs and pilus types for 699 (87.3%) were determined by the use of our pneumococcal typing pipeline with Illumina WGS fastq files provided by the Sanger group ($n = 516$ from year 2009 and 2012 IPD isolates) and the CDC Biotechnology Core Facility ($n = 183$ from year 2013 IPD isolates). The WGS-derived data from these 699 isolates recovered during 2009, 2012 and 2013 were used to supplement the already accumulated conventional serotyping, antimicrobial susceptibility testing, MLST, pilus locus PCR and PCR/ESI-MS data (described below). WGS-based identification of serotypes, pilus loci and non- β -lactam antimicrobial resistance features employed query DNA sequences described in Table S1 (serotypes and pili) and Table S2 (non- β -lactam antimicrobial resistance). Transpeptidase domain amino acid sequences of 277–359 residues from penicillin-binding proteins (PBPs) 1a, 2b, and 2x were extracted from approximately 1600 ABCs strains characterized over the years 1998–2013. From this, databases of 69, 77 and 127 unique transpeptidase domain amino acid sequences were compiled for PBP1a, PBP2b, and PBP2x, respectively (Tables S3–S5). Each unique sequence was assigned an identifier (sequences 1a-0 to 1a-68 for PBP1a, sequences 2b-0 to 2b-78 for PBP2b, and sequences 2x-0 to 126 for PBP2x). The three-number combination from each isolate was correlated with MICs for each of the six different β -lactam antibiotics. For example, the basally β -lactam-sensitive TIGR4 strain (genbank accession AE005672) contains a composite amino acid sequence pattern of 1a-0, 2b-0, and 2x-0 (abbreviated as 0:0:0). The corresponding DNA sequences (e.g. 1a-0, 2b-0, and 2x-0) are italicized. Full-length PBP genes are referred to with standard nomenclature (*pbp1a*, *pbp2b*, and *pbp2x*).

Detection of non-core genome-conferred resistance loci was performed according to homology with known determinants. Core genome-encoded resistance determinants (fluoroquinolones, co-trimoxazole, ribosomal protein mutation-conferred macrolide/lincosamide/streptogramin resistance, and rifampin) were screened by identifying specific amino acid substitutions with previously described targets (Table S2). Table S2 describes the sequence coordinates used for resistance queries. The bioinformatics methods used are described in Doc. S1.

Serotype verification, CC determination and pilus determinant detection for year 2008 and 2011 isolates

Quellung-determined serotypes were verified from IPD isolates ($n = 622$) from 2008 and 2011 by use of the previously described PCR/ESI-MS [9]. CCs were assigned on the basis of known clone and serotype associations with partial to complete

MLST profiles (obtained by Sanger sequencing) and antimicrobial resistance profiles as previously described [8,10]; these CCs were then verified with PCR/ESI-MS. Year 2009 isolates, most of which were also subjected to WGS analysis as described above, were processed in the same manner, except without PCR/ESI-MS. Pilus determinant PCR-based detection was performed with previously described primers [11]. Twenty-five of the year 2008 isolates were also retrospectively subjected to the WGS typing pipeline. Serotypes, pilus determinants and MLSTs obtained with WGS were in complete concordance with the results obtained with the other methods.

Assignment of CC reference MLSTs

Multilocus sequence typing relied upon the database at <http://pubmlst.org/spneumoniae/>. Forty-seven new sequence types (STs) and 21 new MLST alleles from this study were deposited in this database. CCs were defined as sharing four or more alleles with a given reference ST. CC reference STs were chosen according to abundance within a given serotype and/or their presence within multiple serotypes of the study, and differed by at least three alleles from other CC reference STs.

Antimicrobial susceptibility

MICs were determined with the microbroth dilution method as previously described [8]. The antimicrobials tested included penicillin, amoxycillin, meropenem, cefotaxime, ceftriaxone, cefuroxime, erythromycin, clindamycin, co-trimoxazole, tetracycline, chloramphenicol, rifampin, levofloxacin, synercid, linezolid, telithromycin, and vancomycin. MICs were interpreted according to 2013 CLSI guidelines [12], with exceptions for the different β -lactam antibiotics and chloramphenicol. Penicillin susceptibility, intermediate resistance, resistance and high-level resistance were defined as (in mg/L) ≤ 0.06 , 0.12–1.0, 2.0, and ≥ 4 , respectively. The highest MIC observed for cefotaxime and/or ceftriaxone was used as a single MIC value for third-generation cephalosporins (these two antibiotics usually showed the same MIC, and never varied by more than a single dilution), with susceptibility, intermediate resistance, resistance and high-level resistance defined as ≤ 0.5 , 1.0, 2.0, and ≥ 4 , respectively. For amoxycillin, susceptibility, intermediate resistance, resistance and high-level resistance were defined as ≤ 0.12 , 0.25–1, 2.0, and ≥ 4 , respectively. For meropenem, susceptibility, intermediate resistance, resistance and high-level resistance were defined at ≤ 0.25 , 0.5, 1.0, and ≥ 2.0 , respectively. Only chloramphenicol MICs of >8 mg/L were considered to indicate resistance, differing from the CLSI value of ≥ 8 mg/L. Intermediate and full co-trimoxazole (trimethoprim–sulphamethoxazole) resistance was defined as MICs (for trimethoprim–sulphamethoxazole) of 1/19–2/38 mg/L and $\geq 4/76$ mg/L, respectively.

Fastq file accession

Accession information and sequencing methodology details for the 727 fastq files utilized in this work are provided in Table S6.

Results

IPD due to PCV13-targeted serotypes

A total of 3024 isolates were collected through ABCs during 2005–2013 from 3443 cases of IPD in children aged <5 years. The percentage of IPD cases with corresponding isolates ranged from 84% to 91% (Fig. 1). During 2005–2009, overall rates of IPD in children aged <5 years of age remained at 21–23 cases per 100 000 individuals [2], as reflected by 384–443 IPD isolates for each of these years within the surveillance population (Fig. 1). The yearly number of 13-valent pneumococcal conjugate vaccine (PCV13) serotype isolates ranged from 223 to 290, accounting for 58–65% of the total IPD isolates. As recently reported [2], the major causes of IPD in children aged <5 years immediately prior to PCV13 implementation (early 2010) were PCV13 serotypes 19A and 7F. During 2005–2009, the numbers of serotype 19A and 7F isolates for each year were 136–190 and 27–71, respectively, with the largest numbers of isolates for both serotypes being recovered during 2009. The incidence of these serotypes dropped dramatically during 2011–2013, with only 14 serotype 19A isolates and two serotype 7F isolates being recovered during 2013. PCV7 serotypes and serotype 6A cumulatively accounted for only 76 isolates during 2005–2009 (3.7%) and for 15 isolates during 2011–2013 (2.5%). Serotype 19F was the most common PCV7-targeted serotype encountered, with 33 isolates (43.4%) being recovered during 2005–2009 and nine isolates (60%) being recovered during 2011–2013. Throughout the 9-year period, there was a general trend for a decrease in the number of PCV7-targeted serotypes, from a high of 28 isolates recovered during 2005 to a low of four isolates recovered during 2013. There were no isolates recovered of the four PCV7-targeted serotypes 6A, 6B, 9V and 23F during 2011–2013, as compared with 23 recovered during 2005–2009.

Whereas there were a small number of IPD cases due to PCV13 serotypes 1 and 5 during 2005–2009 (29 serotype 1 and five serotype 5 isolates recovered), there were no cases detected during 2010–2012, and there was only one serotype 1 isolate recovered during 2013. The minor paediatric IPD serotype 6C is also predicted to be targeted by PCV13 through cross-protection from the 6A antigen [13], which adds a small increment to the total of potential PCV13-preventable cases (5–12 cases during each of the years 2005–2012; total of 64 isolates). There was no reported serotype 6C paediatric IPD during 2013; however, paediatric serotype 6C IPD in prior

6C not common

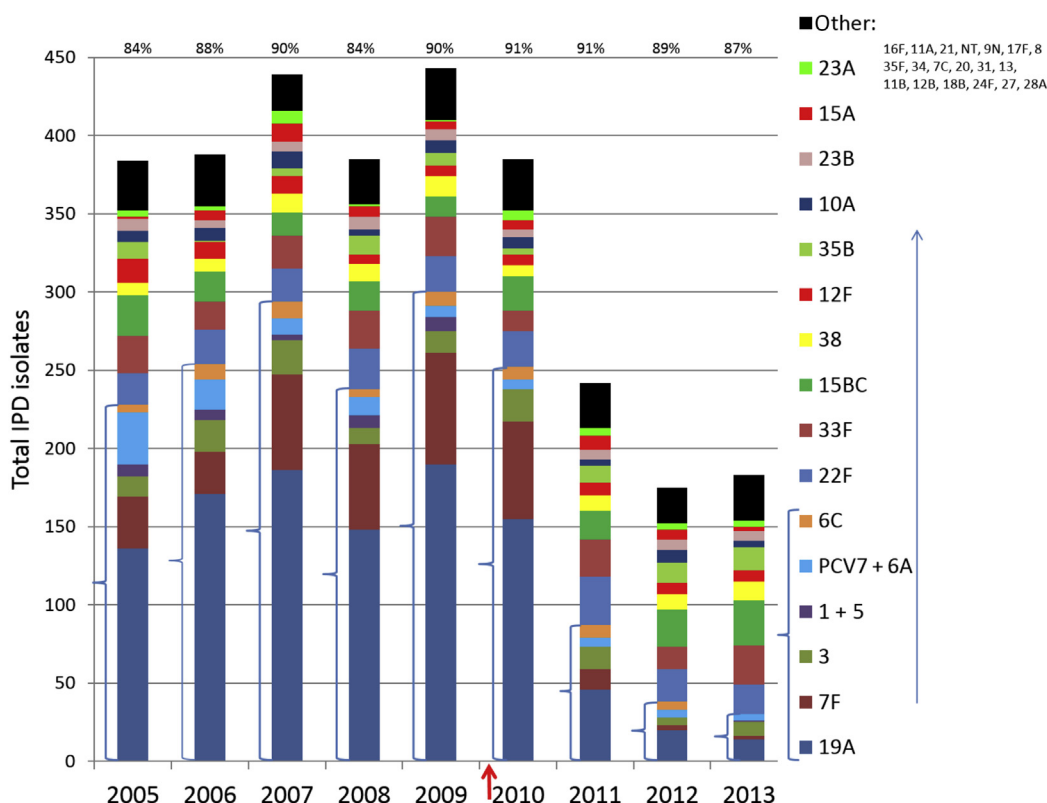


FIG. 1. Invasive pneumococcal disease (IPD) isolate serotype distributions in children aged <5 years during 2005–2013 as identified through Active Bacterial Core surveillance. Thirteen-valent pneumococcal conjugate vaccine (PCV13) serotypes and 6C are bracketed. The arrow indicates PCV13 implementation in February, 2010. The percentages shown above each year indicate the ratios of IPD isolates to documented IPD cases (see <http://www.cdc.gov/abcs/reports-findings/surv-reports.html>). NT, non-typeable; PCV7, seven-valent pneumococcal conjugate vaccine.

years was too rare for the significance of this finding to be calculated.

IPD due to non-PCV13 serotypes

The four most frequent causes of IPD during 2011–2013 were serotypes 33F, 22F, 15B/15C, and 35B; there were cumulatively 244 isolates recovered of these four serotypes during these three years (72–88 isolates per year). Serotypes 38, 12F, 23B, 10A, 15A and 23A combined accounted for 120 isolates during these three years (36–42 each year). Each of these non-vaccine serotypes showed relatively little fluctuation in IPD incidence throughout the entire period (2005–2013), and there were no clear indications of so-called serotype replacement disease. Although the number of 33F IPD isolates nearly doubled in 2013 ($n = 25$) relative to 2012 ($n = 14$), the numbers of serotype 33F IPD isolates recovered were nearly identical during each of the years 2005, 2008, 2009, 2011, and 2013 (Fig. 1). The remaining, less frequently encountered, non-PCV13 serotypes that were encountered during the 2005–2013 period, listed according to frequency (cumulative isolates), were 16F (38), 11A (37), 21 (29), 8 (19), 9N (19), 17F (18), 35F (17), 34 (14), 7C (12), 20 (8),

31 (8), 13 (3), 11B (1), 12B (1), 18B (1), 24F (1), 27 (1), and 28A (1). These serotypes also showed no clear indications of becoming increasingly common after PCV13 introduction, and cumulatively accounted for only 18–31 isolates each year during 2005–2013 (18–28 during 2011–2013). There were 24 total non-typeable (NT) IPD isolates recovered during 2005–2013. Features of the nine NT isolates recovered during 2008–2009 and 2011–2013 are described below.

Serotype and genotype diversity of invasive pneumococci recovered during 2008–2013

A total of 1428 IPD isolates were characterized from the years 2008–2009 and 2011–2013. Fig. 2 is provided to give a comprehensive representation of the CC diversity within each serotype during these 5 years. This work included WGS-based characterization of 699 isolates recovered during 2009, 2012, and 2013 (Table 1), representing 35 of the 39 serotypes that, together with nine NT isolates, accounted for all IPD cases during the 5 years of the study (serotype 6A, 6B, 14 and 20 isolates were not recovered during 2009, 2012, and 2013). The WGS data included WGS-derived capsular serotypes, MLSTs,

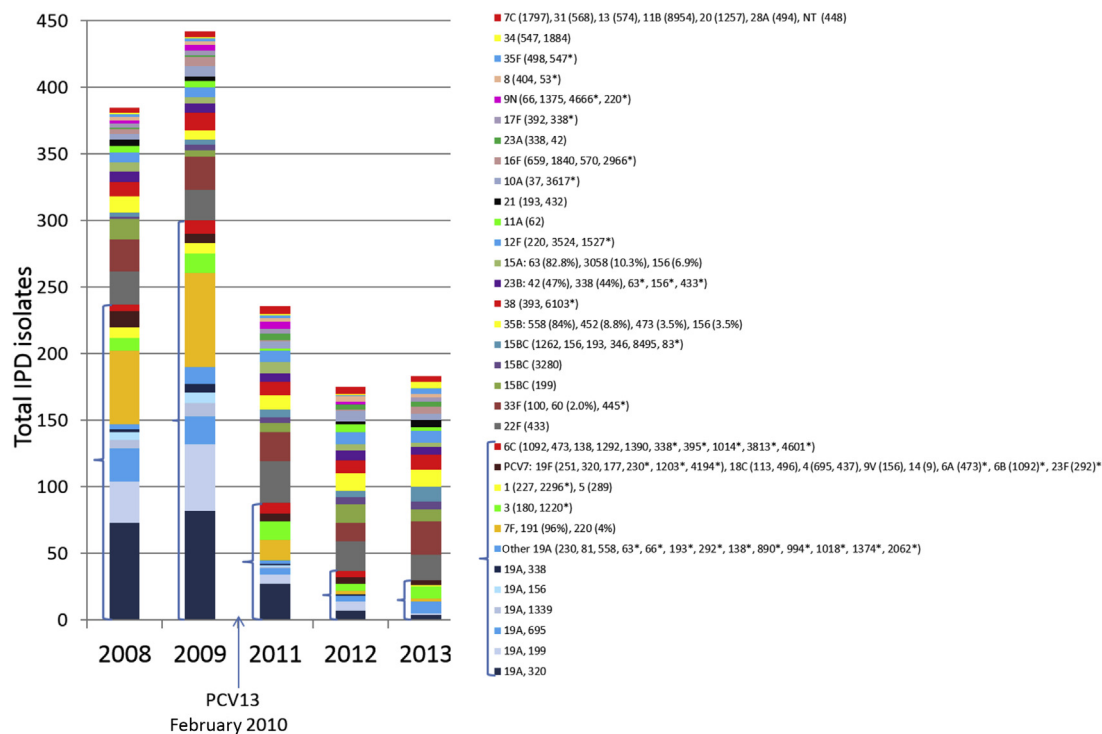


FIG. 2. Comprehensive listing of clonal complex (CC) associations with the serotypes encountered in invasive pneumococcal disease (IPD) isolates during 2008–2009 and 2011–2013. Asterisks indicate CCs that were encountered in single isolates. Thirteen-valent pneumococcal conjugate vaccine (PCV13) serotypes and 6C are bracketed. Certain variants are presented as percentages; for example 7F (191) constitutes 96% of serotype 7F, and 7F (220) constitutes 4%. NT, non-typeable.

transpeptidase protein sequence type patterns, pilus determinant detection, and sequence-based antimicrobial resistance markers (correlated with conventional MIC-based results).

Genetic composition of individual non-vaccine type (NVT) serotypes

Below, we provide selected information regarding specific NVT serotypes of interest from among the 27 total NVTs encountered. We have restricted this text to predominant serotypes and individual variants that potentially reflect past serotype switch events or express unusual resistance properties within given serotypes.

Serotypes 22F and 33F

Serotypes 22F and 33F were each composed primarily of single MLST-based complexes that appear to have been largely unchanged throughout 2008–2013 and in comparison with the year immediately before PCV7 introduction (1999) [14]. Both serotypes uniformly lacked pilus determinants PI-1 and PI-2 (Table 1). As also shown in Table 1, approximately 25% of serotype 22F isolates recovered during 2009, 2012 and 2013 expressed *mef*-associated erythromycin resistance. Most serotype 33F isolates were resistant to erythromycin (*mef*-positive)

and were primarily intermediately resistant to co-trimoxazole. Intermediate co-trimoxazole resistance in these and most other isolates of this phenotype, was associated with one or two codon insertions between bases 168 and 201 of the *folP* structural gene. A small number of serotype 33F isolates were fully co-trimoxazole resistant, and this was associated with both *folP* insertion and the *folA* I100L substitution. The single β -lactam-non-susceptible (NS) serotype 33F strain (phenotype I1SSR) featured a PBP profile (4:23:7) suggestive of previous horizontal transfer(s) of PBP gene sequences from a CC558 donor strain into a common 33F/CC100 strain, as 1a-4 and 2x-7 are found nearly exclusively in β -lactam-NS 35B/CC558 strains (Table 1). Although 2b-23 is unique in our database to this specific serotype 33F isolate, it also appears to have originated from a recombination event with a β -lactam-NS strain, as it contains six substitutions that closely neighbour the active site serine SXXK and SXN. These include three of four substitutions in this region (T426K, Q427L, and T446A) that are associated with intermediate penicillin resistance [15].

Serotypes 15B and 15C

Serotypes 15B and 15C show nearly identical CC distributions (data not shown), owing to their frequent interconversion

(1) Continued on next page

TABLE 1. Continued

Serotype, isolates characterized in years 2009;2012;2013 ^b	CC percentage of the serotype during			ST ^d (relation to reference CC ST) ^e (no. identified)	PI-1, PI-2 (no. when variation)	PBP 1a:2b:2x profile (no. when variation)	β-Lactam R profile ^f (no. when variation)	Non-β-lactam R profile ^g (no. when variation)	Resistance determinant profile ^h (no. characterized as shown ^a)
	Reference CC ST ^c	2009	2012–2013						
11A, 5:6:2	62	100	100	62 (8)	–, + (3) – – (5)	2:6:0 (5) 2:6:38 (1) 2:6:10 (2)	SSSSS	SSSSS RSRSS RRRSS SSISS	Negative (2) mef (2) mef, folA 1100L + folP insertion (1) folA 1100L (1) folP insertion (1)
11B, 0:0:1 12F, 7:7:7	8954 220	0 85.7	1 (100) 100	10154 (SLV) (1) 10153 (DLV) (1) 220 (8)	–, – – – – –	0:6:10 1:0:0 0:0:2 (6) 0:0:17 (4)	SSSSS SSSSS SSSSS SSSSS	SSRSS RRRSS RRRSS SSSSS	folA 1100L + folP insertion (1) mef, folA 1100L + folP insertion (1) mef, folA 1100L + folP insertion (1) Negative (8) mef (1) folP insertion (1)
13, 2:0:0	3776 574	14.3 100	0 0	10171 (DLV) (1) 1176 (TLV) (1) 3776 (1) 574 (1) 9462 (SLV) (1) 63 (4)	–, – – – – – + – + – –, –	0:0:0 Not done 23:4:1 11:0:0 11:0:0 24:27:28 (2) 24:27:35 (1) 24:73:114 (1) 24:27:28 (1) 24:22:28 (2)	SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS	Not done Negative (1) Negative (1) Negative (1) Negative (1) ermB, folP insertion, tetM (2) ermB, tetM (1) ermB, folP insertion, tetM (1) ermB, folP insertion, tetM (3)	
15A, 5:5:3	63	80.0	75.0	9939 (SLV) (3)	–, –	Not done	SSSSS	SSSSS	Not done (1)
15B + 15C, 14:24:28 (14:24:26)	3058 156 199	20.0 0 35.7	0 25.0 47.0	Not done (1) 3811 (SLV) (2) 199 (8)	–, – + – –, –	1:0:0 2:0:2	SSSSS SSSSS	SSSSS SSSSS	Negative (2) Negative (8) mef (1) Negative (2) Negative (2) mef (1) Negative (2) Negative (2) Negative (1) ermB, folP insertion, tetM (10) mef, folP insertion (1)
	3280	28.6	23.5	200 (SLV) (1) 10156 (DLV) (1) 3280 (10)	– – + – (10) – – (1)	0:0:0 7:8:9	SSSSS SSSSS		

tetM (1)
Continued on next page

TABLE 1. Continued

Serotype, isolates characterized in years 2009:2012:2013 ^b	Reference CC ST ^c	CC percentage of the serotype during		ST ^d (relation to reference CC ST) ^e (no. identified)	PI-1, PI-2 (no. when variation)	PBP 1a:2b:2x profile (no. when variation)	β-Lactam R profile ^f (no. when variation)	Non-β-lactam R profile ^g (no. when variation)	Resistance determinant profile ^h (no. characterized as shown ^b)
		2009	2012–2013						
19F, 3:3:2	1339	5.3	0	1339 (3)	+, + (2) –, + (1)	27:30:8	RRHH (2) HHSR (1) HHRH (2) RRHH (1)	RSRS	mef, folA 1100L + folP insertion (3)
				2268 (SLV) (3)		27:36:8	HHRH (2) RRHH (1)	RSRS (2) RSIS (1)	mef, folA 1100L + folP insertion (2) mef, folP insertion
				2270 (SLV) (1)	+, +	27:30:8	RRHH	RSRS	mef, folA 1100L + folP insertion (1)
				8207 (SLV) (1)	+, +	27:11:8	HHRH	RSRS	mef, folA 1100L + folP insertion (1)
				10178 (SLV) (1)	+, +	27:30:8	RRHH	RSRS	mef, folA 1100L + folP insertion (1)
				10159 (DLV) (1)	+, +	27:30:8	RRHH	RSRS	mef, folA 1100L + folP insertion (1)
	156	4.2	0	156 (5)	+, –	29:12:26 (4) 41:12:36 (1)	RRHH HHRH	RSRS	mef, folA 1100L + folP insertion (5)
				4026 (SLV) (2)	+, –	28:14:36	HHRH	RSRS	mef, folA 1100L + folP insertion
				166 (SLV) (1)	+, –	15:12:7	HHRH	RRRS	mef, folP insertion
				10225 (TLV) (1)	+, –	65:38:36	HHRH	RSRS	ermB, folA 1100L + folP insertion, tetM (1)
21, 3:3:5 (3:2:5)	338	3.2	2.9	8197 (DLV) (2) 338 (1)	–, – –, –	17:1:22 0:1:1	ISSS ISSS	RSRS RSRS	mef, folA 1100L, folP insertion (1) folP insertion (2)
	558 ^g	1.1	0	10150 (DLV) (1)	–, –	17:1:22	ISSS	RSRS	Negative (1) folP insertion (1)
	66	0.5	0	66 (1)	+, –	47:7 (2009)	IRSH	RSRS	mef (1)
	81	0.5	0	2346 (SLV) (1)	–, –	47:16 (2006)	RRHH	RRRR	Negative (1) Negative (1)
	138	0.5	0	639 (DLV) (1)	–, –	2:0:0	SSSS	SSSS	ermB, folA 1100L + folP insertion, tetM, cat (1)
	292	0.5	0	292 (1)	–, –	30:0:0	SSSS	SSSS	Negative (1)
	2062	0	2.9	2062 (1)	–, –	16:13:19	ISSS	SRRS	Negative (1)
	63	0.5	0	63	–, –	Not done	SSSS	RRRS	folA 1100L + folP insertion (1)
	1374			1374 (1)	+, +	23:75:126	SSSS	RRRS	ermB, tetM, folP insertion
	320	33.3	40.0	271 (SLV)	+, +	13:14:20 (1) 13:16:47 (1)	RRHH RRHH	RRRS	mef, ermB, folA 1100L + folP insertion, tetM (3)
22F, 23:21:19	251	0	40.0	251 (1) 654 (SLV) (1)	+, – +, –	2:0:0 2:0:0	SSSS SSSS	SSSS	Negative (1) Negative (1)
	177	1 (33.3)	0	179 (SLV) (1)	+, –	34:32:43	RRHH	RRRS	ermB, tetM (1)
	346	0	20.0	1203 (SLV) (1)	–, –	20:18:15	SSSS	SSSS	Negative (1)
	230	33.3	0	Not done (1)	–, –	Not done	SSSS	SSSS	Not done (1)
	432	33.3	71.4	432 (6)	–, –	0:0:2	SSSS	SSSS	Negative (6)
	193	66.7	28.6	193 (2) 3689 (SLV)	–, – –, –	2:0:2	SSSS SSSS	SSSS	Negative (2) Negative (1)
	433	100	97.6	433 (47)	–, –	1:2:2 (42) 1:2:27 (2) 1:2:59 (3)	SSSS SSSS SSSS	SSSS	Negative (44) mef (3)
				7314 (SLV) (3)	–, –	1:2:2	SSSS	SSSS	mef (3)
				9456 (SLV) (1)	–, –	1:0:2	SSSS	SSSS	Negative (1)
				10166 (SLV) (1)	–, –	1:2:2	SSSS	SSSS	Negative (1)
23A, 1:4:4				10164 (SLV) (1)	–, –	1:2:2	SSSS	SSSS	mef (1)
				10165 (SLV) (1)	–, –	1:2:2	SSSS	SSSS	Negative (1)
				10167 (SLV) (1)	–, –	1:2:2	SSSS	SSSS	mef (1)
				10163 (DLV) (3)	–, –	1:2:2	SSSS	SSSS	mef (2)
				10226 (DLV) (1)	–, –	1:2:2	SSSS	SSSS	Negative (1)
	698	0	2.4	698 (1)	–, –	0:2:0	SSSS	SSSS	mef (1)
	338	100	75.0	338 (7)	–, –	0:1:1 (5) 19:1:24 (1) 1:1:48 (1)	ISSS (6) ISSS (1)	SSIS (3)	folA 1100L (2) folP insertion (1)
				10147 (SLV) (1)	–, –	2:0:6	SSSS	SSSS	mef (1)
				1839 (DLV) (1)	–, –	12:0:0	SSSS	SSSS	ermB, folA 1100L + folP insertion (1)
	42	0	25.0		–, –			SSSS	Negative (1)

23B, 7:7:7 (7:6:7)	338	42.9	38.5	1373 (DLV) (6)	–, –	0:1:1 (5) 21:1:1 (1) 7:21:1 (1) 7:1:1 (1) 0:0:0 (4) 0:0:11 (1) 0:24:29 (1) 0:0:119 (1)	SSISS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS	folP insertion (3) Negative (3) mef, folP insertion (1) folA 1100L + folP insertion (1) Negative (6) mef (1)
	42	42.9	46.1	439 (SLV) (6)	–		SSSSS	
23F, 1:0:0 28A, 0:0:1 31, 0:2:1 33F, 25:14:27 (25:14:25)	156	0	7.7	33 (DLV) (1) 1847 (DLV) (1) 162 (SLV) (1) 63 (1)	–, – –, – +, –	12:0:2 0:0:0 0:0:0	SSSSS SSSSS SSSSS SSSSS	Negative (1) Negative (1) Negative (1) ermB, tetM (1)
	433 292 494 568 100	14.3 0 100 0 92.0	0 7.7 0 1 (100) 3 (100) 100	4334 (TLV) (1) 2520 (TLV) (1) 494 (1) 568 (2) 100 (14)	–, – –, – –, – –, – –	24:27:28 0:2:0 12:68:104 2:2:2 12:0:15 2:0:6 (12) 2:20:6 (1) 64:0:6 (1)	SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS	Negative (1) Negative (1) Negative (1) folA 1100L + folP insertion, tetM (1) Negative (1) Negative (2) folP insertion (6) mef, folP insertion (6) folA 1100L + folP insertion (1) mef (1)
34, 1:1:5	60 1012 547	4.0 4.0 100	0 0 83.3	2705 (DLV) (34)	–, –	2:0:6 (34)	SSSSS	mef, folP insertion (30) mef, folA 1100L + folP insertion (3) mef (1) folP insertion (1) mef, folP insertion (1) Negative (1) ermB, tetM (2)
	1884 558	0 62.5	16.6 92.3	9029 (SLV) (2) 717 (TLV) (2) 7507 (SLV) (1) 10145 (DLV) (1) 10168 (DLV) (1) 10142 (TLV) (1) 10169 (DLV) (1) 10146 (TLV) (1) 60 (1) 1012 (1) 547 (5) 10157 (SLV) (1) 1884 (1) 558 (17)	–, – –, – –, – –, – –, – –, – –, – –, – –, – –, – –, – –, – +, –	2:0:6 (1) 2:9:6 (1) 2:0:6 (1) 2:72:6 (1) 2:0:6 (1) 2:0:6 (1) 4:23:7 2:0:6 (1) 2:0:6 2:0:6 2:4:2 2:0:2 0:0:2 0:0:2 4:7:7 (16) 4:7:112 (1) 4:19:7 (1)	SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS (17) RSISS (1)	folP insertion (1) mef, folP insertion (1) Negative (1) ermB, tetM (2) mef, folP insertion (1) folP insertion (1) mef, fol insertion (1) mef, folP insertion (1) mef, folA 1100L + folP insertion (1) mef, folP insertion (1) Negative (1) Negative (1) Negative (5) Negative (1) Negative (1) mef (17) mef, folA 1100L (1)
35B, 8:13:15 (8:13:13)	473 156	25.0 12.5	0 3.9	10222 (SLV) (1) 10175 (SLV) (1) 3822 (SLV) (1) 10174 (SLV) (1) 156 (1)	+, – +, – –, – +, –	4:7:7 4:14:7 23:4:21 4:7:18 4:12:7	RSISS RSISS SSSSS SSSSS SSSSS	mef (1) mef (1) Negative (1) mef, folA 1100L + folP insertion (1) mef, folA 1100L + folP insertion (1)
	452 498	0 100	3.9 100	10158 (SLV) (1) 498 (3) 1635 (DLV) (2) 393 (27)	–, – –, – –, – –	0:0:0 0:3:3 0:3:3 2:4:4 (19) 2:4:4 (4) 2:44:1 (1) 0:0:2 (1) 2:4:0	SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS SSSSS	Negative (1) Negative (1) Negative (1) Negative (1) Negative (1) Negative (3) Negative (26) Negative (26) folP insertion (1)
35F, 2:1:4	393	100	100	1140 (DLV) (1)	–, –		SSSSS	Negative (1)

Continued on next page

Continued on next page

TABLE 1. Continued

Serotype, isolates characterized in years 2009:2012:2013 ^b	Reference CC ST ^c	CC percentage of the serotype during		ST ^d (relation to reference CC ST) ^e	PI-1, PI-2 (no. when variation)	PBP 1a:2b:2x profile (no. when variation)	β-Lactam R profile ^f (no. when variation)	Non-β-lactam R profile ^g (no. when variation)	Resistance determinant profile ^h (no. characterized as shown ^a)
		2009	2012–2013						
NT ⁱ , 2:2:0	448 1292 1480 199	0 1 0 0	1 0 0 1	448 (1) Not done (1) 1480 (1) 199 (1)	– – – –	Not done Not done 3:5:5 2:0:2	SSSSS SSSSS SSSSS SSSSS	SSSSS SSSSS SSSSS SSSSS	Not done (1) Not done (1) Negative (1) Negative (1)

CC, clonal complex; DLV, double-locus variant; H, high-level resistance; I, intermediate resistance; PBP, penicillin-binding protein; R, resistance; S, susceptibility; SLV, single-locus variant; ST, sequence type; TLV, triple-locus variant.

^aFeatures of a serotype 19A/ST558 strain recovered during 2006 [19] are also shown.

^bWhole genome sequence (WGS)-based data are in columns 1, 5, 6, 7, and 10. There was a perfect correlation between Quelling-based and WGS-based serotype determination (column 1), except for three of the four non-typeable isolates at the bottom, for which the indicated serotype-specific signatures were found from genomic sequences (see footnote 1). The numbers for which serotypes and the other indicated parameters were determined with WGS as well as with Quelling are indicated in the last column shown. To simplify the table, minimal data are added for isolates characterized solely through conventional methods and PCR/electrospray ionization mass spectrometry. Such data are only included when the CC within the serotype is not represented by WGS-based data. For this reason, 13 of the 722 isolates are described as 'not done' for multiple WGS-based parameters. For these isolates, serotypes, CCs, partial or complete multilocus sequence types, resistance features and plus detection were obtained solely with conventional (non-WGS-based) methods.

^cNumbers of isolates within each serotype characterized are provided for each year, and abridged numerical methods are provided in parentheses when not all of the isolates of the indicated serotype were available. The ratios of isolates within indicated serotypes during the 3 years are only approximately proportional to the overall invasive pneumococcal disease incidence fluctuation of the serotype.

^dReference CC is chosen on the basis of the overall representation within multiple serotypes or the predominance within a specific serotype. Each CC has at least three non-identical alleles with other reference STs shown in the table. Individual STs within a CC must share at least four alleles with the reference ST.

^eAll STs designated 10142 or higher were initially found during this study.

^fIndicated when the ST is related to the reference CC as an SLV, DLV, or TLV.

^gThe order of β-lactam R phenotypes is as follows: 1, penicillin; 2, amoxycillin; 3, meropenem; 4, cefotaxime/ceftriaxone (whichever is the highest); and 5, cefuroxime. The phenotype abbreviations and corresponding MIC values are provided in Materials and methods.

^hThe order of non-β-lactam R phenotypes is as follows: erythromycin, clindamycin, co-trimoxazole, tetracycline, and chloramphenicol. MIC ranges (mg/L) are described in Materials and methods.

ⁱFor serotype 19A/CC199, the various R phenotypes and causal determinant signatures shown in the last two columns are correlated by the use of identical symbols (!, +, *, #, or @). Phenotypes that are discordant with the determinant listing in column 10 are indicated in bold.

^jThe WGS-based serotype assignments for these Quelling-non-typeable isolates are as follows for the following genotypes: ST448, non-serotypeable; CC1292, serotype 6C; ST199, serotype 15B; ST1480, serotype 8.

based on variation within *wciZ15B* [16]. Of the 185 combined serotype 15B and 15C isolates recovered during 2005–2013, 115 (62%) were serotype 15C. The ability to resolve these two serotypes was included within our typing pipeline, owing to the reported low cross-reactivity of functional antibodies against serotype 15B with serotype 15C [17], which is a consideration in the formulation of new vaccines. These two serotypes accounted for 84 isolates for the years for which we provided genotyping (2008–2009 and 2011–2013), and showed a high level of genetic heterogeneity as compared with the pre-PCV7 years, when ABCs serotype 15B/15C isolates were primarily comprised of CC199 [14]. Although CC199 was the single most common serotype 15B/15C CC, numerous additional CCs as a group constituted up to 65% of these two serotypes (Fig. 2), with CC3280 being the most frequent secondary CC. As shown in Table 1, CC3280 has markedly more resistance features than the other common serotype 15B/C CCs (resistance to macrolides and co-trimoxazole; low-level β-lactam resistance). Several other CCs were evident at low frequencies, including CC156. CC156, which is characteristic of the major serotype 9V strain that flourished prior to PCV7 implementation [14], featured a number of single-locus variants within serotypes 15A, 15B/15C and 23B that, unlike their serotype 9V counterparts, were uniformly susceptible to β-lactams and generally susceptible to other antibiotics (Table 1).

A single highly resistant serotype 15C strain was a single-locus variant (ST83) of ST81, which is the genotype of the highly successful multiresistant PMEN-I [18]. This strain showed moderate to high resistance to all five β-lactam classes, and was resistant to the five additional antimicrobials listed in Table 1. This ST83 strain shared PBP profile 15:12:18, which was also found within a multiresistant serotype 19A/ST2346 strain (ST81 single-locus variant) and two serotype 9V/ST156 strains in this study (Table 1). The 15:12:18 profile is also shared among numerous pre-PCV7 IPD isolates of serotypes 9V, 19F and 23F within CC81 and CC156 in our master pneumococcal genome database (data not shown). Prior to this study, ST83 was associated in the global MLST database solely with strains collected in Asian countries during 1995–2013 that primarily included serotype 23F strains, but also included serotype 15B and 15C strains.

Serotype 35B

Serotype 35B was primarily comprised of the serotype 35B ST558 complex during all 5 years, which slightly increased in frequency during 2011–2013 (Fig. 2). This strain complex is typically associated with the presence of pilus PI-1 and elevated MICs for penicillin and other β-lactam antibiotics (Table 1; Figs. 4 and 5). It is interesting that, during the pre-PCV7 years, serotype 35B strains rarely caused IPD in children, and the now

uncommon β -lactam-sensitive serotype 35B complex (CC452, one isolate depicted in Table 1) accounted for approximately 31% of 35B IPD isolates from patients of all ages [19]. Two additional interesting CCs were observed within serotype 35B. A single-locus serotype 35B variant of ST473 (ST3822; also described in Table 1) was recovered during 2009. CC473 has been previously associated with serotypes 6A and 6C [10], and ST3822 has been recorded from a single serotype 6A blood isolate recovered during 2008 in Ireland (<http://pubmlst.org/spneumoniae/>). Because of PBP allele and MLST associations, the two serotype 35B/CC156 isolates (Table 1) appear to be progeny of serotype switch events, and are described in more detail in the section below pertaining to capsular locus switching.

Serotype 6C

The most genetically heterogeneous NVT serotypes were serotypes 6C and 15B/15C (already described). Although it caused a low level of IPD in children aged <5 years (five to nine isolates during each of the years 2008–2009 and 2011–2012), serotype 6C was composed of two different CCs, four of which were represented by single isolates (Fig. 2; Table 1). The absence of recovered serotype 6C IPD isolates during 2013 possibly reflects the previously predicted PCV13 cross-protection against serotype 6C [13].

We have verified that all of the depicted CCs shown for serotype 6C (Fig 2), except for CC3813, have been previously associated with serotypes 6A and 6B, including CC473 found in the single serotype 6A isolate and CC1092 from the single serotype 6B isolate recovered during the 5 years of the study (both recovered during 2008) [7,10]. No serotype 6A-mediated or serotype 6B-mediated IPD was observed during 2009–2013 in children aged <5 years, presumably because of the protection of PCV7 (during 2009) and PCV13 (during 2012–2013). Serotype switching events targeting the *cps*-internal *wciN* genes that distinguish serotypes 6A and 6C appear to have occurred many times between the different serogroup 6 lineages [7].

Serotype 15A

Most of these isolates were within CC63, which is characteristic of the global PMEN clone PMEN-23 (<http://www.pneumogen.net/pmen/>). This CC gradually became predominant within ABCs serotype 15A during 2001–2007 [10]. CC63 strains contained highly related PBP patterns that were generally associated with intermediate resistance to penicillin and amoxycillin, with the exception of the single 24:73:114 pattern, which was associated with intermediate resistance to cefotaxime and ceftriaxone, and high-level resistance to cefuroxime. CC63 isolates were nearly uniformly resistant to erythromycin, clindamycin, and tetracycline (*ermB* and *tetM* positive), with *folA*

and/or *folP* mutations conferring co-trimoxazole non-susceptibility (Table 1).

Two ST3811 (ST156 single-locus variant) type 15A isolates were recovered in the post-PCV13 period, presumably reflecting a past serotype switching event between a serotype 15A donor and a CC156 recipient strain. Both isolates were sensitive to antibiotics and featured the PBP 1:0:0 profile, which is associated with basal β -lactam sensitivity (see serotypes 4, 9N and 11B and 15B/C for additional examples of this widely shared profile in Table 1). All of the various CC156 clones described in this study (Table 1) retain pilus PI-1 positivity that is characteristic of the presumed serotype 9V/ST156 ancestral strain. The major serotype 9V complex CC156, which was predominant in the pre-PCV7 years [14], was associated with six additional serotypes besides serotype 9V in this study (Table 1), including 15A, 15B, 15C, 19A, 23B, and 35B.

Of the CC156 non-9V strains, only the serotype 19A and 35B variants expressed levels of β -lactam resistance similar to that of serotype 9V/ST156. The serotype 19A/ST156 and serotype 19A/ST166 strains each share 2b-12, which is also found in the 9V/ST156 strain, with the 19A/ST166 strain additionally carrying the CC156-associated 1a-15.

Serotypes 23A and 23B

It is interesting that these two serotypes are completely (23A) or primarily (23B) associated with the two same CCs (CC338 and CC42). Although they feature different predominant STs within CC338, strains of both serotypes within CC338 show similar antimicrobial resistance features (Table 1).

Serotype 23B variants of CCs that are thought to have originated within other serotypes were additionally recovered (one or two isolates each of CC156 (9V), CC63 (15A), and CC433 (22F)).

Serotype 17F

Serotype 17F isolates were associated with CC392 genotypes as in the pre-PCV7 years, with the exception that a single ST338 isolate, which was intermediately penicillin resistant and a presumed serotype switch variant, was observed. ST338 is a primary genotype of US serotype 23A strains, is a minor serotype 19A genotype (Table 1), and has been reported in the past as a major serotype 23F clone [20].

NT IPD isolates

As was recently reported, isolates apparently lacking a capsule were rare among strains causing IPD [21], consistent with the capsule being required for establishing most invasive infections. Of the nine NT isolates recovered over the entire 5-year period, seven were of clonal clusters and *cps* locus signatures typical of commonly occurring serotypes, including 19A/

CC695, 15B/CC199, 6C/CC1390, 6C/CC1292, 7F/CC191, and 8/CC1480. Although the *cps* loci of these nine isolates were not examined in detail for this study, our recent population-based findings with NT IPD isolates from patients of all ages recovered during 2006–2009 indicated that 90% of such isolates contained *cps* loci, with serotype-specific *cps* locus signatures and MLST types being highly associated with known serotypes [21]. Within the 801 isolates representing 2009, 2012, and 2013, there were only four NT isolates (Table 1). The study isolates from the 5 years included two NT ST448 isolates (recovered during 2008 and 2012) that are of a distinct pneumococcal lineage highly associated with carriage and conjunctivitis. This lineage lacks capsular biosynthetic genes, and has evolved with divergent virulence and adhesion factors [22].

Clonal distributions of PCV13 serotypes

Subset PCV7. PCV7 serotypes showed high genetic diversity, considering their very small numbers throughout the time period (Table 1; Fig. 2).

For 2009, 2012, and 2013, PCV7-targeted serotypes accounted for a total of 16 isolates of serotypes 4, 9V, 18C, 19F, and 23F. Serotype 19F accounted for half of these isolates within five different CCs that each accounted for one to three isolates (Table 1).

Serotype 19A. The frequency of serotype 19A IPD decreased by approximately 90% in 2012 and 2013 relative to 2009 (Fig. 1), with all year 2009 CCs being either greatly decreased in incidence or not found (Fig. 2). It is noticeable, however, that the PCV7 escape variant 19A ST695 complex that was originally discovered among early post-PCV7 IPD isolates [5,6] and that spread throughout the US during subsequent years [8,23] showed a disproportionally higher residual IPD incidence in 2013 than the other serotype 19A complexes (Fig. 2). Three rare variants were observed within serotype 19A within CC138, CC2062, and CC558. To our knowledge, serotype 19A/CC138 strains have not been previously described, as this complex has been largely restricted to serotype 6B [14]. Serotype 19A/CC2062 represents numerous isolates recovered recently in South Africa (see <http://pubmlst.org/spneumoniae/>). Isolates within CC138 and CC2062 were not detected in a comprehensive analysis of year 2005–2007 ABCs serotype 19A isolates [8,24]; however, a single serotype 19A/ST558 isolate was recovered during 2006 [8]. The two serotype 19A/558 variants described here (from 2006 and 2009) appear to indicate capsular locus switching from serotype 35B to serotype 19A, and are described in more detail below.

Serotype 7F. The second most abundant IPD serotype during 2008–2009, serotype 7F, was composed almost exclusively of highly uniform (PI-2 positive, antimicrobial susceptible) ST191

isolates (Fig. 2; Table 1), and its incidence declined to nearly non-detectable levels during 2013 (only two isolates recovered).

Other PCV13 serotypes

The serotype 3/ST180 complex was associated with a small number of cases throughout 2008–2009 and 2012–2013 (5–14 isolates each year). A single ST1220 isolate was recovered during 2012.

The relatively rare IPD serotypes 1 and 5 each mainly represented single CCs, although, as shown in Table 1, there was considerable MLST variability within a small sample of the PI-2 positive CC227. The single outlier genotype ST2296 was from the single serotype 1 isolate recovered during 2013. There were no serotype 1 isolates recovered during 2011–2012.

Identification of donor, recipient and progeny strains in serotype switch events by the use of MLST and PBP data

The two serotype 19A/CC695 variants 19A/ST695 and 19A/ST2365 were initially discovered and described as vaccine escape variants in the post-PCV7 years [5,24], and were both later characterized at the genomic sequence level [6,23]. Genomic analysis of several independent isolates of these variants was performed. In addition, several independent isolates of the deduced serotype 19A/CC199 donor strains and serotype 4/ST695 recipient (recipient 1 in Fig. 3A) [6] were also identified, although a serotype 4 recipient strain corresponding to serotype 19A/ST645 donor strain 2 and serotype 19A/ST2365 progeny strain 2 was not found among ABCs surveillance isolates. Genomic comparisons of multiple isolates of the donor, recipient and progeny strains depicted in Fig. 3A revealed that each of the two progeny strains arose through an independent multifragment recombination event from serotype 19A/CC199 donor strains [6,23]. The published *pbp1a* and *pbp2x* sequences from the putative serotype 19A/ST199 donor and serotype 4/ST695 recipients in the previous study [6] were identical within their overlaps with the entire *1a-8* and *2x-11* sequences found in all 22 19A/ST695 isolates recovered during 2009, 2012, and 2013, and also in a single serotype 19A/ST199 isolate (8:29:11) recovered during this period (Table 1). The 8:29:11 PBP profile is also represented in multiple serotype 19A/ST199 isolates in our pre-PCV7 ABCs genomic database (not shown). The sensitive PBP pattern 0:0:0 was predominant (14 of 20 isolates) among serotype 4/ST695 isolates in our database from 1998 to 1999 (whereas serotype 4/ST695 IPD isolates were common during this time, serotype 19A/ST695 isolates were not found [10,25]). Thus, it is likely that *2x-11*, *cps19a* and *1a-8* were transferred and integrated into a serotype 4/ST695 recipient to concurrently facilitate a serotype 4 to

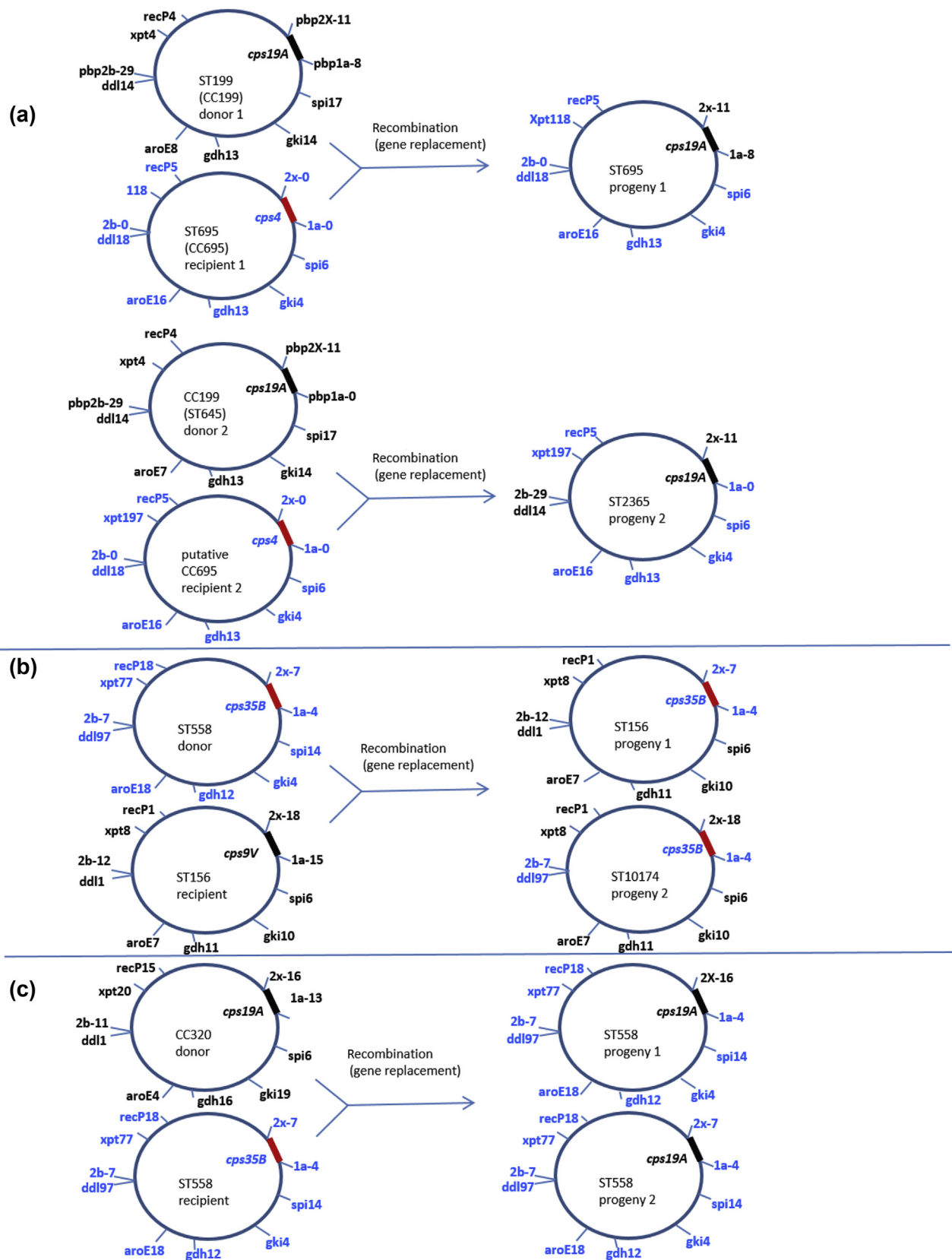


FIG. 3. (a). Diagrammatic representations of independent and potentially PCV7 vaccination driven serotype switch involving PCV13 serotype donors (19A) and PCV7 serotype (4) recipient. (b) Two independent potential vaccine-escape events with non-vaccine serotype donor (35B) and PCV7 serotype recipient (9V). (c). Two independent serotype switch events involving non-vaccine type (35B) recipient and PCV13 type donor(s). Putative donor, recipient and progeny strains were deduced from multilocus sequence type and penicillin-binding protein profiles. Markers in the progeny that are shared with the donor share the same colour font, which differs from that in the recipient. CC, clonal complex; ST, sequence type.

serotype 19A switch and reduced β -lactam susceptibility (penicillin MICs of 0.06–0.12 mg/L) [6]. We observed that the *1a-0* and *2x-0* sequences from the serotype 4/ST695 isolates are identical to the sensitive TIGR4 strain, and that the donor and progeny 1 *1a-8* and *2x-11* contain mosaic sequences with substitutions previously associated with β -lactam resistance [26,27].

In the previous study [6], another serotype 19A/CC199 (ST645, a single-locus variant of ST199) strain was identified as the likely donor in a second independent serotype 4 to serotype 19A switch. Although a CC695 recipient strain was not identified, the preponderance of serotype 19A/CC199 isolates with a 0:29:11 PBP profile (Table 1) and the observation of this same profile in serotype 19A/ST2365 (progeny 2) are indicative of co-replacement of the *cps4* and flanking *2x* marker of a recipient CC695 strain with the donor *cps19A* and *2x-11*. This is also consistent with the single observed unlinked recombination event whereby the closely linked CC695 *ddl18* (exclusively found within serotype 4/CC695 strains in our database) and *2b-0* chromosomal segment was replaced by the corresponding DNA fragment from serotype 19A/CC199 containing *ddl14* and *2b-29* (Fig. 3A) [23]. We believe that the serotype 19A/ST2365 progeny 2 strain has reduced penicillin susceptibility (we have observed these strains to have MICs of 0.06–0.12 mg/L) owing to the *2x-0* to *2x-11* replacement event. The putative unlinked replacement of *2b-0* with *2b-29* is not expected to contribute to β -lactam resistance, given the lack of key substitutions within *2b-29* associated with resistance [15], even though these two *pbp2b* transpeptidase regions vary in six of 278 residues. The progeny 2 variant 19A/ST2365 shares the complete 0:29:11 profile with the serotype 19A/CC199 donor (Fig. 3A). These two different recombination events represent two of the previously depicted nine fragments within serotype 19A/ST2365 progeny strains that did not match known serotype 4/CC695 recipient genomes and matched a common serotype 19A/CC199 putative donor strain [23].

Another likely example (Fig. 3B) of pneumococcal conjugate vaccine escape capsular switching is shown by two putative independent switch events involving serotype 35B/ST558 and serotype 9V/ST156 as donor and recipient, respectively. The serotype 35B/ST156 (progeny 1 strain in Fig. 3B) retained all seven ST156 MLST alleles and the *2b-12* characteristic of this complex; however, *1a-4* and *2x-7* were apparently co-integrated with *cps35B* into this progeny 1 strain during the double-crossover serotype replacement event. In the progeny 2 strain (serotype 35B/ST10174), it appears that the region encompassing *cps9V* and its flanking *1a-15* was replaced by *cps35B* and its flanking *1a-4*, whereas *2x-18* flanking the opposite end of *cps9V* was unaffected during this replacement event. As in the unlinked replacement event described in the

Fig. 3A progeny 2 strain, a second, unlinked, double-crossover event seems likely to have occurred within this progeny strain, resulting in replacement of the CC156 *ddl-1* and neighbouring *2b-12* with *pbp2b-7* and *ddl-97*. Besides this serotype 35B/ST10174 variant, the *ddl-97* allele has been exclusively associated with ABCs 35B/CC558 strains during the past 20 years (data not shown).

A third example of a likely serotype switch strain set (donor, recipient, two progeny strains) is shown in Fig. 3C (serotype 35B to serotype 19A). Both serotype 19A/ST558 isolates are depicted in Table 1 and in Fig. 3C. Each isolate has two or three of the PBP alleles within pattern 4:7:7, and susceptibility profiles that are found in most common serotype 35B/ST558 isolates (Table 1). As in the case with the putative serotype 9V to serotype 35B replacement event depicted in Fig. 3A, the differing PBP profiles of the progeny strains are suggestive of two independent serotype switch events: one involving co-replacement of *cps35B* and *2x-7* with *cps19A* and *2x-16* (progeny 1, recovered during 2006), and the other solely involving replacement of *cps35B* with *cps19A* (progeny 2, recovered during 2009). Although the predominance of *2x-16* within the serotype 19A/CC320 strain (Fig. 1) that was highly prevalent during the pre-PCV13 period is strongly suggestive of its role as a donor strain in this switch event, the PBP pattern data of the progeny 2 serotype 19A strain does not provide evidence of the 19A donor strain genotype.

Removal of strains positive for pilus determinants and resistance genes by continued vaccination

Concurrently with the dramatic decrease in the incidence of IPD that occurred in the period 2011–2013, there were decreases in the incidence rates of strains genetically positive for all three categories of pilus determinants, including PI-1-positive strains, PI-2-positive strains, and strains positive for both PI-1 and PI-2 (Fig. 4). The incidence of strains positive for only PI-1 decreased by 32–55% in 2008–2009 relative to 2011–2013, primarily because of the removal of the serotype 19A/ST695 and serotype 19A/ST156 strains. A small increase in the incidence of the PI-1 positive NVT serotype 35B complex was observed. The incidence of strains positive for PI-2 decreased even more dramatically post-PCV13, owing to the decrease in the incidence of the serotype 7F/ST191 complex. Serotype 19A/ST320 accounted for almost all of the isolates that were positive for both PI-1 and PI-2 throughout all years, and was the sole positive strain detected during 2013 after a decrease of approximately 97% relative to 2009. The residual strains positive for both PI-1 and PI-2 during 2011–2012 were also of PCV13 serotypes, including serotype 19A/ST1339 and the rarely occurring PCV7 serotype 19F strains that are highly related to 19A/ST320 (see Table 1 for strain features).

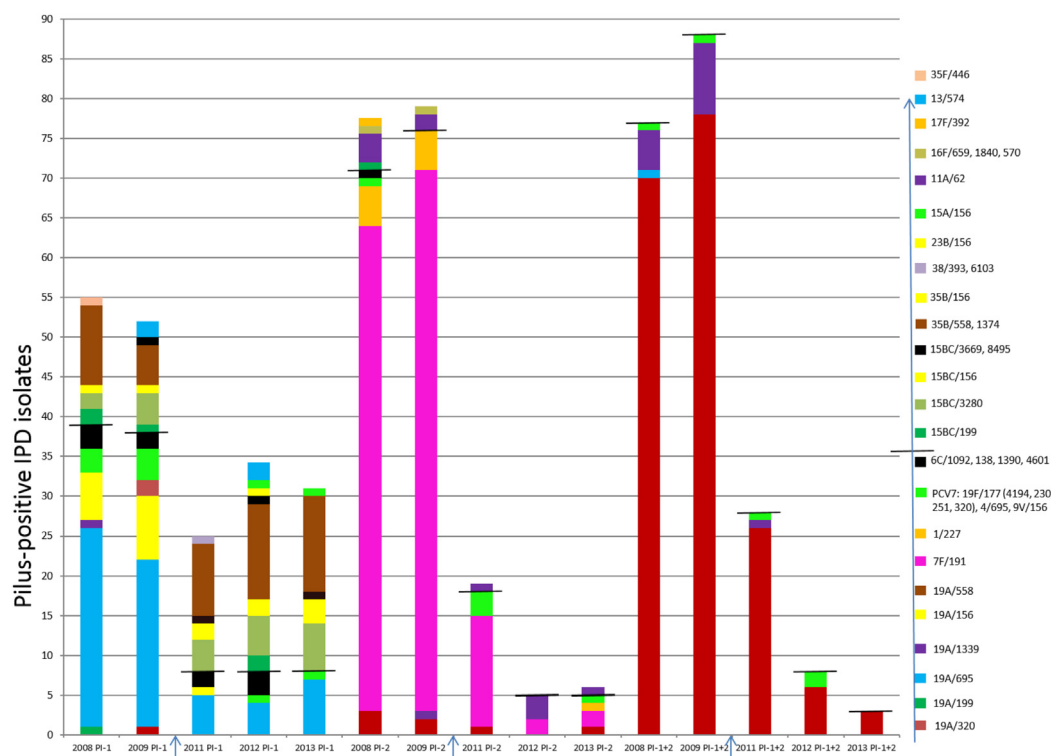


FIG. 4. Decreases in the incidence rates of invasive pneumococcal disease (IPD) isolates positive for pilus determinants associated with decreases in pneumococcal clonal complexes after 13-valent pneumococcal conjugate vaccine (PCV13) implementation (2011–2013) in early 2010 (arrows). Bars 1–5 and 6–10 from the left indicate isolates positive for only PI-1 and PI-2, respectively. Bars 11–15 indicate isolates positive for both PI-1 and PI-2. A horizontal line on each bar indicates the cut-off between PCV13 serotype plus 6C strains (below the line) and non-PCV13 serotype strains (above the line). PCV7, seven-valent pneumococcal conjugate vaccine.

The major overall contributor to all of the 11 categories of resistance throughout the period 2008–2009 was serotype 19A/CC320 (Fig. 5). Resistance during this period became more common among pneumococci, because of a combination of additional serotype 19A CCs and several other residual PCV7 CCs. The major contributor to the penicillin resistance phenotype (with and without erythromycin resistance) during 2012–2013 was serotype 35B/CC558, which showed a moderate increase relative to previous years. Serotype 33F/CC100 became the predominant erythromycin-resistant/clindamycin-sensitive IPD strain complex during 2013. The incidence of isolates that were erythromycin resistant (owing to *mef* and/or *ermB* determinants) decreased by 56–60% during 2012–2013 relative to 2009, whereas the clindamycin resistance conferred by *ermB* decreased by 81–87% (*ermB* co-confers erythromycin resistance in almost all strains). Other resistance phenotypes showed similar dynamics, with serotype 19A/CC320 and other PCV13 CCs declining, leaving non-PCV13-targeted serotypes as the residual contributors to resistance. The last six combinations of resistance depicted in Fig. 5 were largely eliminated through removal of serotype 19A/CC320, with no indications

of new emergent complexes. The only strains that were resistant to all six antimicrobials depicted in Fig. 5 (penicillin, cefotaxime and/or ceftriaxone, erythromycin, clindamycin, cotrimoxazole and tetracycline) during 2008–2013 were serotype 19A/CC320 (many isolates) serotype 19A/CC81 (four recovered during 2008–2009), serotype 19F/CC320 (one recovered during 2012), and serotype 15C/CC81 (one recovered during 2013). Representatives of these strain sets are described in Table 1.

Resistance determinants

Among the 1428 isolates from the 5 years of the study, there was no observed resistance to fluoroquinolones, rifampin, linezolid, telithromycin, quinupristin–dalfopristin, or vancomycin. Of these 1428 isolates, 724 were subjected to WGS analysis and our automated algorithm for detecting specific resistance determinants. Only two of the 104 instances of combined resistance to erythromycin and clindamycin were not attributable to *ermB* (Table 1). In one (serotype 6C/ST138), the A2061G substitution, which is known to confer resistance to macrolides and lincosamides (Table S2), was

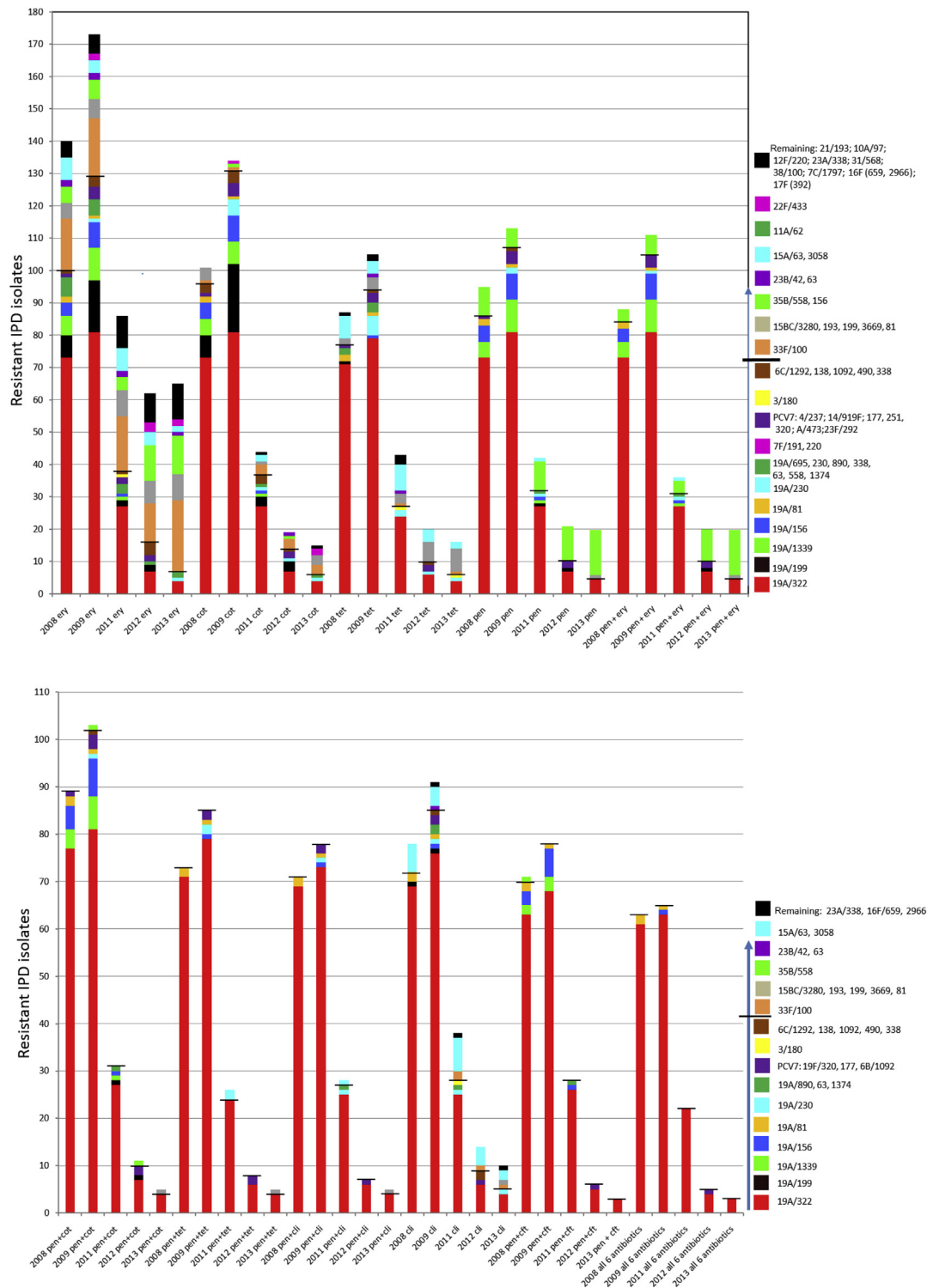


FIG. 5. Decreases in antimicrobial resistance to six different antimicrobials and their combinations associated with decreases in the incidence rates of pneumococcal clonal complexes after 13-valent pneumococcal conjugate vaccine (PCV13) implementation in 2010. The horizontal line on each bar indicates the cut-off between PCV13 serotype plus 6C strains (below the line) and non-PCV13 serotype strains (above the line). Note that intermediate resistance is not included. co-trimoxazole; ery, erythromycin; pen, penicillin; tet, tetracycline; cli, clindamycin; cft, cefotaxime.

detected. In the second, no causal mechanism was detected (serotype 19A/ST1756). In one isolate (serotype 19A/ST320), *ermB* was detected; however, the isolate was clindamycin sensitive (Table 1). The erythromycin-resistant/clindamycin-susceptible and chloramphenicol-resistant phenotypes in these isolates were entirely accounted for by the *mef* (166 instances) and *cat* (four instances; see serotypes 3/CC180, 15BC/CC81, 19A/CC81, and 16F/CC2966) determinants, respectively (Table 1). Tetracycline resistance was associated with *tetM* in all instances; however, three of the 134 *tetM*-positive isolates were tetracycline sensitive (two serotype 19A/ST320 and one serotype 15A/ST63). Intermediate to full co-trimoxazole resistance was always attributable to mutations within one or both of *folA* and *folP*. Most instances (107/112) of intermediate resistance were due to insertions within a hot-spot of *folP*, with the other five instances being attributed to the well-known *folA* I100L substitution (Table S2). Full co-trimoxazole resistance was attributed to two unlinked mutations (*folP* insertion and *folA* I100L) in 159 of 160 instances (Table 1). In one resistant isolate, only a *folP* insertion was observed (serotype 19A/ST10160).

Predicting β -lactam MICs from PBP patterns

The utility of composite PBP transpeptidase domain profiles as an additional genetic and phenotypic marker in tracking recombination events is apparent (see above). These profiles are also useful for rapidly assessing MICs for different β -lactam antibiotics. For example, 83 isolates in Table 1 that represented seven different CCs shared profile 2:0:2 and were basally β -lactam sensitive. There were numerous additional instances of basally sensitive profiles, several of which were very common. There was no instance of a basally sensitive profile being seen among strains showing resistance or reduced susceptibility to β -lactams. As expected, most PBP profiles associated with β -lactam resistance were highly associated with specific CCs. The association of eight basally sensitive CC156 variants (presumably descendants of the highly resistant serotype 9V/ST156 strain) with two common sensitive profiles (0:0:0 and 1:0:0) is consistent with the notion that clonally associated genetic determinants other than PBP transpeptidase-encoding sequences may not commonly play dominant roles in β -lactam resistance phenotypes. Also consistent with the dominant role of PBP transpeptidase regions in conferring β -lactam resistance is the observation that PBP pattern 15:12:18 was solely found among β -lactam-resistant strains of both CC156 and CC81 (serotype 9V/ST156, serotype 19A/ST2346, and serotype 15B/ST83) that shared closely similar β -lactam MICs (data not shown). Within given PBP patterns, there was little deviation evident in the β -lactam resistance profiles for the five different classes of antibiotics shown. When profiles SSSSS and ISSSS were shared

within isolates sharing a PBP profile (e.g. 0:29:11, shared by many serotype 19A/CC199 isolates), the penicillin MICs for susceptible and intermediate isolates were always 0.06 mg/L and 0.12 mg/L, respectively. Similar observations of a one dilution difference were generally observed throughout these data when both intermediate and resistant, or both resistant and highly resistant, MIC results for the different β -lactams were observed for isolates sharing the same PBP transpeptidase profiles (data not shown).

Discussion

The most important strain characteristics tracked by IPD strain surveillance have not changed in decades. At present, the most critical features required for vaccine evaluation and planning and treatment recommendations are still the capsular serotypes and antimicrobial susceptibilities. Nonetheless, population-based assessment of invasive pneumococcal strain features at a deeper level within individual serotypes can potentially detect undesirable developments within this very adaptable species that could undermine prevention efforts. For example, although this work provides reassuring evidence that the various lineages of the genetically complex serotype 19A appear to have either disappeared or to cause only a small residual level of IPD, it is conceivable that an antigenic variant of serotype 19A could escape being targeted by PCV13. It would be logical that such a variant would be initially detectable as a minor genetic lineage within the serotype that shows an IPD incidence that is inconsistently elevated as compared with other lineages of the same serotype. Serotype 6C is an example of a serotype 6A immunological variant that escaped notice for decades, owing to its serological similarity to serotype 6A [28]. The detection of serotype 6C with monoclonal antibody-based methodology [28] allowed the use of simple PCR-based methods [7] to easily discern serotype 6C from serotype 6A, and to allow serotype 6C inclusion for retrospective and prospective surveillance. This serotype was almost never detected in children prior to PCV7 implementation; however, it became readily detectable, although not common, in paediatric IPD patients after PCV7 implementation. Serotype 6C also became a commonly carried serotype in children [29], and has become a common cause of IPD in adults [2]. The lack of serotype 6C IPD isolates recovered in this study during 2013 from the paediatric population gives hope that a protective effect might eventually be seen in adults.

The invasive serotype 35B is presently composed primarily of one CC that is potentially poised to take advantage of reductions in the frequencies of other strains in the carriage reservoir that are occurring in the post-PCV13 era. In some

ways, this can be compared with the serotype 19A situation immediately prior to PCV7 implementation. Like the most frequent serotype 19A IPD-causing CC during 1999 (CC199), the major serotype 35B/CC558 strain set caused a moderate level of IPD during the pre-PCV13 period, was β -lactam NS, and has shown indications of increased carriage during recent years [29]. It is now conceivable that, as the major β -lactam NS CC in the nasopharyngeal reservoir, and with the removal of most PCV7 and PCV13 serotype competitors, serotype 35B/CC558 and perhaps other variants, such as serotype 35B/CC156, will thrive. Although the serotype 35B/CC156 variant is very rare, we did not detect serotype 19A/CC320 strains, despite extensive genotypic surveillance of paediatric ABCs isolates during 1999 and 2001 [14,25]; yet this complex became the single most common cause of IPD among people of all ages during the mid-2000s to late 2000s [8]. It is with some concern that two independent invasive serotype 35B/CC156 isolates (ST156 and its single-locus variant ST9910) have already been recovered during ongoing year 2015 IPD strain surveillance (data not shown). The serotype 19A/CC695 complex, which originated after PCV7 implementation from independent serotype 4 to serotype 19A switch events [5,6,8,23,24], also emerged significantly in a very short period after PCV7 implementation [8]. The two serotype 35B/CC156 strains described in this study are likely to be independent immune escape variants (serotype 9V to serotype 35B) that occurred after PCV7 implementation. In contrast, the two putative instances of serotype switch that involved replacement of the NVT *cps35B* locus with the PCV13 type *cps19A* locus (Fig. 3C) are consistent with naturally occurring intraspecies transfers of capsular biosynthetic and PBP genes, which were shown to be widespread between commonly occurring strains before conjugate vaccine implementation [30].

It is interesting that four of the current non-vaccine serotypes appeared to concurrently undergo a clonal shift during the years between the implementation of PCV7 and the implementation of PCV13 [10]. Each of serotypes 15A, 35B, 23A and 6C were primarily associated with penicillin-susceptible IPD isolates during 1999–2001. Within each of serotypes 15A, 35B, and 23A, single penicillin-NS CCs (serotypes 15A/CC63, CC35B/CC558, and 23A/CC338) gradually became predominant during subsequent post-PCV13 years [10]. Serotypes 15B and 15C showed increased antimicrobial resistance and genetic complexity as compared with the pre-conjugate vaccine era, and is potentially poised to significantly emerge in the post-PCV13 era. It remains to be determined whether post-PCV13 clonal shifts will occur within the two predominant non-vaccine serotypes, 22F and 33F, that are each primarily represented by single β -lactam-susceptible CCs.

The primary purpose of this work was to provide a thorough descriptive reference for important features of IPD strains afflicting our paediatric population. The secondary aim was to implement a more informative and efficient method for the routine determination of serotypes, resistance features, pilus determinants, and genotypes. WGS-based serotype deduction was successful because there is remarkable sequence conservation within the *cps* loci for individual serotypes. An example of this conservation is given by the *wcwD7F* locus used for distinguishing serotype 7F from serotype 7A, where inactivating mutations distinguish the rarely occurring serotype 7A from serotype 7F. Of the 75 Quellung-typed serotype 7F isolates examined, 74 shared a perfectly conserved 9630bp *wcwD7F*, and one contained a single missense (A25V) base substitution. We feel that harnessing our first version of an automated WGS pipeline allows us to monitor the changes within IPD strains that are primarily driven by horizontal genetic transfer events at a much deeper and detailed level. For example, although serotype switch events can readily be identified within isolate sets through performing multilocus sequence typing and serotyping, the examination of switch variants in more detail will allow better elucidation of donor lineages, and identification and tracking of unlinked determinants that are often simultaneously co-transferred during these events. Genomic analyses of pneumococcal strains indicate that the term 'serotype switch' is an oversimplification, and that it often involves simultaneous recombination events at multiple unlinked chromosomal loci [4,18,23].

Removing the primary IPD-causing serotypes concurrently removed from the pneumococcal population strain reservoirs for resistance and adhesion that are likely to serve as advantages in carriage. It follows that additional advantageous features within the species are removed or depleted from the carriage reservoir through this strategy. It is hoped that the durability of the protection afforded by PCV13 will approximate that of PCV7, which, as compared with the pre-vaccination years, reduced the incidence of paediatric IPD throughout the 2000s by approximately 80% [2,3].

Transparency declaration

The authors declare that they have no conflicts of interest.

Acknowledgements

Major funding for this work was provided through support from the Advanced Molecular Detection (AMD) initiative at CDC. This work was supported in part by the Gates Foundation Grant OPP1034556 to Emory University and the Sanger

Institute core grant (Wellcome Trust Grant 098051), which funded half of the genome sequencing costs. We thank J. Jorgensen and his laboratory group for antimicrobial susceptibility testing of all isolates other than those recovered in Georgia and Minnesota during the period 2005–2012. We are grateful to the Minnesota Department of Public Health laboratory for pneumococcal serotyping and susceptibility testing of all isolates recovered in Minnesota. We thank the following individuals for their contributions to the establishment and maintenance of the ABCs system. California Emerging Infections Program: A. Reingold, S. Brooks, and H. Randel. Colorado Emerging Infections Program: L. Miller, B. White, D. Aragon, M. Barnes, and J. Sadlowski. Connecticut Emerging Infections Program: S. Petit, M. Cartter, C. Marquez, and M. Wilson. Georgia Emerging Infections Program: M. Farley, S. Thomas, A. Tunali, and W. Baughman. Maryland Emerging Infections Program: L. Harrison, J. Benton, T. Carter, R. Hollick, K. Holmes, and A. Riner. Minnesota Emerging Infections Program: R. Lynfield, C. Holtzman, R. Danila, and K. MacInnes. New Mexico Emerging Infections Program: K. Scherzinger, K. Angeles, J. Bareta, L. Butler, S. Khanlian, R. Mansmann, and M. Nichols. New York Emerging Infections Program: N. Bennett, S. Zansky, S. Currenti, and S. McGuire. Oregon Emerging Infections Program: A. Thomas, M. Schmidt, J. Thompson, and T. Poissant. Tennessee Emerging Infections Program: W. Schaffner, B. Barnes, K. Leib, K. Dyer, and L. McKnight. CDC: R. Gierke, K.-A. Toews, E. Weston, L. McGlone, and G. Langley. This publication made use of the *Streptococcus pneumoniae* MLST website (<http://pubmlst.org/spneumoniae/>) sited at the University of Oxford (Jolley & Maiden 2010, *BMC Bioinformatics*, 11:595). The development of this site has been funded by the Wellcome Trust. The findings and conclusions in this report are those of the authors, and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.cmi.2015.08.027>

References

- [1] Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, Lynfield R, et al. Decline in invasive pneumococcal disease after the introduction of protein–polysaccharide conjugate vaccine. *N Engl J Med* 2003;348:1737–46.
- [2] Moore MR, Link-Gelles R, Schaffner W, Lynfield R, Lexau C, Bennett NM, et al. Effect of use of 13-valent pneumococcal conjugate vaccine in children on invasive pneumococcal disease in children and adults in the USA: analysis of multisite, population-based surveillance. *Lancet Infect Dis* 2015;301–9.
- [3] Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* 2010;201:32–41.
- [4] Wyres KL, Lamberts LM, Croucher NJ, McGee L, von Gottberg A, Liñares J, et al. Pneumococcal capsular switching: a historical perspective. *J Infect Dis* 2013;207:439–49.
- [5] Pai R, Moore M, Pilishvili T, Gertz RE, Whitney CG, Beall B. Post vaccine genetic structure of *Streptococcus pneumoniae* serotype 19A from children in the United States. *J Infect Dis* 2005;192:1988–95.
- [6] Brueggemann AB, Pai R, Crook DW, Beall B. Vaccine escape recombinants emerge after pneumococcal vaccination in the United States. *PLoS Pathog* 2007;3:e168.
- [7] Carvalho Mda G, Pimenta FC, Gertz Jr RE, Joshi HH, Trujillo AA, Keys LE, et al. PCR-based quantitation and clonal diversity of the current prevalent invasive serogroup 6 pneumococcal serotype, 6C, in the United States in 1999 and 2006 to 2007. *J Clin Microbiol* 2009;47:554–9.
- [8] Beall BW, Gertz RE, Hulkower RL, Whitney CG, Moore MR, Brueggemann AB. Shifting genetic structure of invasive serotype 19A pneumococci in the United States. *J Infect Dis* 2011;203:1360–8.
- [9] Massire C, Gertz Jr RE, Svoboda P, Levert K, Reed MS, Pohl J, et al. Concurrent serotyping and genotyping of pneumococci by use of PCR and electrospray ionization mass spectrometry. *J Clin Microbiol* 2012;50:2018–25.
- [10] Gertz Jr RE, Li Z, Pimenta FC, Jackson D, Juni BA, Lynfield R, et al. Increased penicillin-nonsusceptibility of nonvaccine serotype (other than 19A and 6A) invasive pneumococci in post 7 valent conjugate vaccine era. *J Infect Dis* 2010;201:770–5.
- [11] Zähler D, Gudlavalletti A, Stephens DS. Increase in pilus islet 2-encoded pili among *Streptococcus pneumoniae* isolates, Atlanta, Georgia, USA. *Emerg Infect Dis* 2010;16:955–62.
- [12] Clinical and Laboratory Standards Institute (CLSI) (2013). Performance standards for antimicrobial susceptibility testing: Twenty-third informational supplement. CLSI document M100-S22. CLSI, Wayne.
- [13] Cooper D, Yu X, Sidhu M, Nahm MH, Fernsten P, Jansen KU. The 13-valent pneumococcal conjugate vaccine (PCV13) elicits cross-functional opsonophagocytic killing responses in humans to *Streptococcus pneumoniae* serotypes 6C and 7A. *Vaccine* 2011;29:7207–11.
- [14] Gertz Jr RE, McEllistrem MC, Boxrud DJ, Li Z, Sakota V, Thompson TA, et al. Clonal distribution of invasive pneumococcal isolates from children and selected adults in the United States prior to 7-valent conjugate vaccine introduction. *J Clin Microbiol* 2003;41:4194–216.
- [15] Contreras-Martel C, Dahout-Gonzalez C, Martins Ados S, Kotnik M, Dessen A. PBP active site flexibility as the key mechanism for beta-lactam resistance in pneumococci. *J Mol Biol* 2009;387:899–909.
- [16] van Selm S, van Cann LM, Kolkman MA, van der Zeijst BA, van Putten JP. Genetic basis for the structural difference between *Streptococcus pneumoniae* serotype 15B and 15C capsular polysaccharides. *Infect Immun* 2003;71:6192–8.
- [17] Rajam G, Carlone GM, Romero-Steiner S. Functional antibodies to the O-acetylated pneumococcal serotype 15B capsular polysaccharide have low cross-reactivities with serotype 15C. *Clin Vaccine Immunol* 2007;14:1223–7.
- [18] Wyres KL, Lamberts LM, Croucher NJ, McGee L, von Gottberg A, Liñares J, et al. The multidrug-resistant PMEN1 pneumococcus is a paradigm for genetic success. *Genome Biol* 2012;13:R103.
- [19] Beall B, McEllistrem MC, Gertz Jr RE, Boxrud DJ, Besser JM, Harrison LH, et al. Emergence of a novel penicillin-nonsusceptible, invasive serotype 35B clone of *Streptococcus pneumoniae* within the United States. *J Infect Dis* 2002;186:118–22.
- [20] Pai R, Gertz RE, Whitney CG, Beall B. Clonal association between *Streptococcus pneumoniae* serotype 23A, circulating within the United

- States, and an internationally dispersed clone of serotype 23F. *J Clin Microbiol* 2005;43:5440–4.
- [21] Park IH, Geno KA, Sherwood LK, Nahm MH, Beall B. Population-based analysis of invasive nontypeable pneumococci reveals that most have defective capsule synthesis genes. *PLoS One* 2014;9:e97825.
- [22] Valentino MD, McGuire AM, Rosch JW, Bispo PJ, Burnham C, Sanfilippo CM, et al. Unencapsulated *Streptococcus pneumoniae* from conjunctivitis encode variant traits and belong to a distinct phylogenetic cluster. *Nat Commun* 2014;5:5411–9.
- [23] Golubchik T, Brueggemann AB, Street T, Gertz Jr RE, Spencer CC, Ho T, et al. Pneumococcal genome sequencing tracks a vaccine escape variant formed through a multi-fragment recombination event. *Nat Genet* 2012;44:352–5.
- [24] Moore MR, Gertz Jr RE, Woodbury RL, Barkocy-Gallagher GA, Schaffner W, Lexau C, et al. Population snapshot of emergent *Streptococcus pneumoniae* serotype 19A in the United States, 2005. *J Infect Dis* 2008;197:1016–27.
- [25] Beall B, McEllistrem MC, Gertz Jr RE, Wedel S, Boxrud DJ, Gonzalez AL, et al. Pre- and postvaccination clonal compositions of invasive pneumococcal serotypes for isolates collected in the United States in 1999, 2001, and 2002. *J Clin Microbiol* 2006;44:999–1017.
- [26] Contreras-Martel C, Job V, Di Guilmi AM, Vernet T, Dideberg O, Dessen A. Crystal structure of penicillin-binding protein 1a (PBP1a) reveals a mutational hotspot implicated in beta-lactam resistance in *Streptococcus pneumoniae*. *J Mol Biol* 2006; 27:355:684–96.
- [27] Gordon E, Mouz N, Duée E, Dideberg O. The crystal structure of the penicillin-binding protein 2x from *Streptococcus pneumoniae* and its acyl-enzyme form: Implication in drug resistance. *J Mol Biol* 2000;299: 477–85.
- [28] Park IH, Moore MR, Treanor JJ, Pelton SI, Pilishvili T, Beall B, et al. Differential effects of pneumococcal vaccines against serotypes 6A and 6C. *J Infect Dis* 2008;198:1818–22.
- [29] Lee GM, Kleinman K, Pelton SI, Hanage W, Huang SS, Lakoma M, et al. Impact of 13-valent pneumococcal conjugate vaccination on *Streptococcus pneumoniae* carriage in young children in Massachusetts. *J Pediatr Infect Dis Soc* 2014;3:23–32.
- [30] Coffey TJ, Dowson CG, Daniels M, Zhou J, Martin C, Spratt BG, et al. Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. *Mol Microbiol* 1991;5:2255–60.