Vaccine Escape Recombinants Emerge after Pneumococcal Vaccination in the United States

Angela B. Brueggemann[1*], Rekha Pai[2], Derrick W. Crook[3], Bernard Beall[4]

1 Department of Zoology, University of Oxford, Oxford, United Kingdom, 2 Department of Gastrointestinal Sciences, Christian Medical College, Vellore, India, 3 Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, United Kingdom, 4 Centers for Disease Control, Atlanta, Georgia, United States of America

The heptavalent pneumococcal conjugate vaccine (PCV7) was introduced in the United States (US) in 2000 and has significantly reduced invasive pneumococcal disease; however, the incidence of nonvaccine serotype invasive disease, particularly due to serotype 19A, has increased. The serotype 19A increase can be explained in part by expansion of a genotype that has been circulating in the US prior to vaccine implementation (and other countries since at least 1990), but also by the emergence of a novel "vaccine escape recombinant" pneumococcal strain. This strain has a genotype that previously was only associated with vaccine serotype 4, but now expresses a nonvaccine serotype 19A capsule. Based on prior evidence for capsular switching by recombination at the capsular locus, the genetic event that resulted in this novel serotype/genotype combination might be identifiable from the DNA sequence of individual pneumococcal strains. Therefore, the aim of this study was to characterise the putative recombinational event(s) at the capsular locus that resulted in the change from a vaccine to a nonvaccine capsular type. Sequencing the capsular locus flanking regions of 51 vaccine escape (progeny), recipient, and putative donor pneumococci revealed a 39 kb recombinational fragment, which included the capsular locus, flanking regions, and two adjacent penicillin-binding proteins, and thus resulted in a capsular switch and penicillin nonsusceptibility in a single genetic event. Since 2003, 37 such vaccine escape strains have been detected, some of which had evolved further. Furthermore, two new types of serotype 19A vaccine escape strains emerged in 2005. To our knowledge, this is the first time a single recombinational event has been documented in vivo that resulted in both a change of serotype and penicillin nonsusceptibility. Vaccine escape by genetic recombination at the capsular locus has the potential to reduce PCV7 effectiveness in the longer term.

Introduction

Streptococcus pneumoniae (the "pneumococcus") is one of the most important bacterial pathogens worldwide, especially among children. Pneumococcal pneumonia, meningitis, and septicemia result in 1 million deaths annually among children <5 y of age [1]. PCV7 protects against seven pneumococcal capsular types (serotypes)—4, 6B, 9V, 14, 18C, 19F, and 23F [2]—and has been used to vaccinate children in the United States since 2000. PCV7 has been remarkably effective in reducing disease among vaccinated children, and even among unvaccinated children and adults as a result of a striking herd immunity effect, due to the disruption of pneumococcal transmission from young children to older children and adults [2–6]. PCV7 is increasingly being used to vaccinate children in other countries.

There are 91 known pneumococcal serotypes, the last of which was discovered recently [7,8]. The ecological niche for the vast majority of the pneumococcal population is the nasopharynx of healthy children [9]; thus, any serotype-specific vaccine that is of limited valency and affects nasopharyngeal carriage will perturb the composition of the circulating pneumococcal population, with unknown consequences. The capsule is the principal known virulence factor with respect to invasive pneumococcal disease [10], and population biology studies indicated that certain serotypes have a greater potential to cause invasive disease than others

[11,12]. Another important aspect of pneumococcal biology is that there is a strong association between genotype as defined by multilocus sequence typing (MLST), and serotype—that is, strains with the same MLST sequence type (ST) are usually of the same serotype [11,13,14].

Since the capsule is the principal invasive disease determinant, and is the target for serotype-specific prevention of disease by vaccination, there are two events that are especially important with respect to understanding vaccine effects: serotype replacement and capsular switching. At a population level, serotype replacement simply refers to a decrease in the prevalence of vaccine serotype pneumococci in the nasopharynx accompanied by a corresponding increase in nonvaccine serotype pneumococci, as they fill the newly

Author Summary

The 7-valent pneumococcal conjugate vaccine is a remarkable public health success story. It has significantly reduced invasive pneumococcal disease in the United States not only by protecting vaccinated children, but also by protecting unvaccinated older children and adults by herd immunity. However, there was always a concern that use of a limited-valency vaccine would result in an increase in disease due to nonvaccine serotypes, and this has now occurred in the US. The predominant nonvaccine serotype causing invasive disease is 19A, and this increase is partially explained by "vaccine escape" pneumococci, strains that have exchanged the genes that encode a vaccine serotype 4 capsule for genes that encode a nonvaccine serotype 19A capsule. These strains are then able to escape vaccine-induced immunity. Characterisation of the genetic event that resulted in these vaccine escape strains was the focus of our study and the results were surprising. The results of this study have important relevance to the long-term effectiveness of the current vaccine and to the development of future pneumococcal vaccines.

vacant ecological niche. Serotype replacement in the nasopharynx of a healthy child may or may not be problematic; the public health concern is whether or not replacement serotypes also cause *disease*. Serotype replacement invasive disease among children and adults has significantly increased in the US post-vaccination [15–18], and invasive pneumococcal disease among children <5 y of age is now predominantly due to serotype 19A [17]. A recent *JAMA* report described a significant overall increase in nonvaccine serotype disease among Native Alaskan children, most frequently due to serotype 19A [18]. Genotypic characterisation of these serotype 19A strains by MLST showed that most of the serotype 19A replacement disease can be explained by clonal expansion of one genotype, ST199, which existed prior to vaccination [15,18].

The second major vaccine-related concern is the possibility of capsular switching, when the genes encoding one type of capsule are exchanged, via transformation and recombination, for the genes encoding a different type of capsule. Capsular switches from one vaccine serotype to another were first described 16 y ago [19–21], but it is the vaccine-to-*nonvaccine* serotype switch that is of primary concern, because it contributes to serotype replacement and allows for the possibility of vaccine escape. Acquisition of a nonvaccine capsule by a pneumococcal strain capable of causing invasive disease has been a serious concern related to the use of any serotype-specific vaccine [11,22].

The capsular locus of the pneumococcus is located between two genes, *dexB* and *aliA* ([7]; Figure 1). (Serotype 37 is one exception, as it has a defective capsular locus and the serotype is determined by the type 37 synthase gene [*tts*] located elsewhere in the pneumococcal genome [7,23].) Two of the six penicillin-binding proteins (PBPs) possessed by the pneumococcus are near the capsular locus: *pbp2x* is upstream of *dexB* and *pbp1a* is downstream of *aliA*. Alterations in PBPs confer penicillin resistance, and alterations in *pbp2x*, *pbp1a*, and *pbp2b* are the most important [24–26]. Penicillin-resistant pneumococci are a major problem throughout the world [27]; prior to use of PCV7 in the US, nearly one-quarter of invasive pneumococci were penicillin-nonsusceptible [28]. Initially, use of PCV7 in the US reduced the incidence of invasive

disease due to antimicrobial-resistant vaccine serotypes, but more recently, antimicrobial resistance has increased with the increase of serotype 19A disease [28,29].

The Centers for Disease Control and Prevention (CDC) has been monitoring invasive pneumococcal disease since 1995 through the Active Bacterial Core (ABC) surveillance program [6,14,30] and as a result, the post-vaccination increase in nonvaccine serotype 19A disease in the US was quickly detected. Serotype 19A strains collected by the CDC through 2005 were genotyped by MLST, which revealed that vaccine escape strains had begun to emerge in 2003 [14,15]. These strains possessed an MLST genotype, ST695, that had always been associated with vaccine serotype 4 (ST695^4), but now expressed a serotype 19A capsule (ST695^{19A}). These strains were detected only 3 y after vaccine implementation, but rapidly increased in prevalence. The first three strains were detected in 2003; two strains were detected in 2004; and 32 strains were detected in 2005, some of which had evolved further. Moreover, in 2005, two new types of serotype 19A vaccine escape strains emerged, ST2365^{19A} ($n = 4$) and ST899^{19A} ($n=1$); these appeared to represent new recombinational events that also occurred between serotype 4 recipients and serotype 19A donors. The aim of this study was to sequence the regions upstream and downstream of the capsular locus, including both PBPs, to identify the putative recombinational event(s) that resulted in these vaccine escape strains.

Results

Recipient, Donor, and Progeny Pneumococcal Strains Selected for Characterisation

Strains were selected based upon serotyping and MLST genotyping data and are described in Table 1. Between 1998 and 2005, 31,669 cases of invasive pneumococcal disease among all ages were identified from ABC sites, from which 28,363 pneumococcal strains were available for serotyping. 15,736 (55%) strains were of nonvaccine serotypes, and 1,935 of those were serotype 19A, 88% of which were recovered from 2000 to 2005 ([17] and ABC surveillance program, unpublished data). The following serotype 19A strains were genotyped by MLST: 82 of 113 (73%) strains from 1999 [14,31] and 779 of 1,597 (49%) strains from 2001 to 2005 ([14,15] and ABC surveillance program, unpublished data). No serotype 19A strains from 1998 or 2000 were genotyped by MLST. Possible serotype 19A capsular switches were identified among 57 of 779 (7%) of 2001–2005 genotyped strains: ST271, 690, 899, 1092, and 1790 ($n = 1$ strain each); ST236, 338, and 557 ($n = 2$ strains each); ST230 and 292 ($n = 3$ strains each); ST156 ($n = 4$ strains); and ST695 ($n = 37$ strains) [14,15,31]. Among these strains, the ST695^{19A} plus related ST899^{19A} strains were characterised in this study. All other possible capsular switch strains were present in very low numbers and/or were also found outside the US; vaccine and nonvaccine serotype variants of ST156 are recognised in the global database (http://www.mlst.net/).

Recipient strains were those with the background genotype into which the 19A capsular locus recombined: ST695^4 ($n=3$), recovered in 1999, and ST899^4 ($n = 1$), recovered in 2002. Based upon pulsed-field gel electrophoresis, selected MLST data, and eBURST analysis, it is likely that strains of ST899^4 were also common among children <5 y of age before vaccine
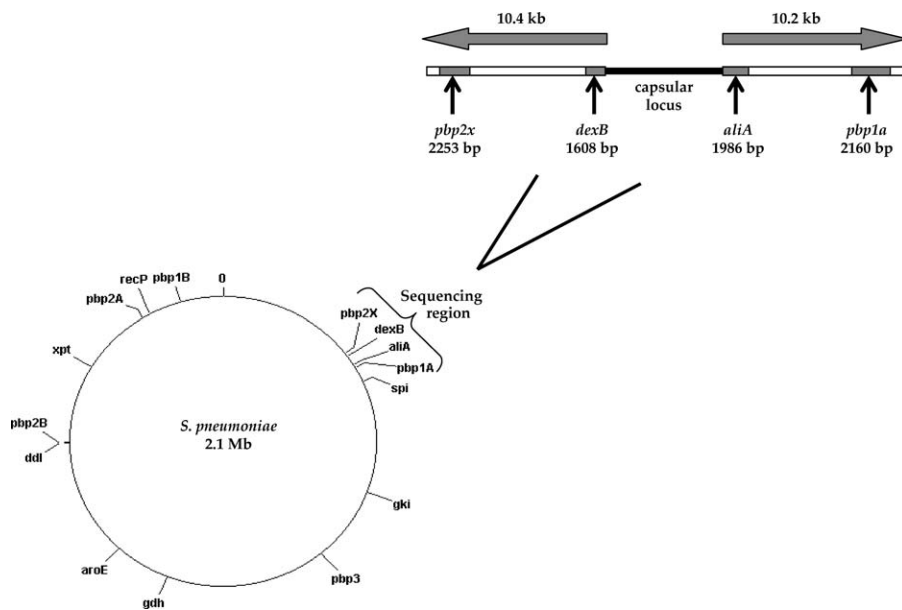
Figure 1. Schematic of the Pneumococcal Genome

Housekeeping genes characterised in the MLST scheme (*aroE, gdh, gki, recP, spi, xpt,* and *ddl*), the genes for PBPs (*pbp2x, -1a, -3, -2b, -2a,* and *-1b*), the capsular locus flanked by the *dexB* and *aliA* genes, and the region around the capsular locus that was sequenced in this study (shown also in expanded version) are depicted. The capsular loci of serotypes 4 and 19A are 20.9 kb and 18.6 kb, respectively, in length.

doi:10.1371/journal.ppat.0030168.g001

introduction [14]. Putative donor strains of the 19A capsular locus were selected from pneumococci recovered in 1999 and 2003. The most likely donor of the 19A capsular locus was ST199[19A], because ST199[19A] existed as a prevalent strain pre-vaccine implementation and was the major clone responsible for serotype replacement disease post-vaccine implementation [15,17]. Thus, four pre- and post-vaccine implementation strains of ST199[19A] were selected, plus one strain of ST645[19A], which is a single-locus MLST variant of ST199[19A]. All serotype 19A strains collected through 2005 that were ST695 or closely related MLST genotypes were characterised as progeny strains. This resulted in the characterisation of 42 unique strains: ST695[19A] ($n = 36$), ST2365[19A] ($n = 4$), ST2363[19A] ($n = 1$), and ST899[19A] ($n = 1$).

Pneumococci were recovered from patients aged <1 to 96 y with invasive pneumococcal disease (Table 1). All pneumococci were recovered from blood, apart from one strain (cdc46) that was recovered from cerebrospinal fluid. Among vaccine escape strains, 40 of 42 (95%) were collected in the northeastern US (note that 37% of all 20,196 available isolates since 2000 were from the Northeast; ABC surveillance program, unpublished data); and two strains were from Minnesota (cdc27) and Colorado (cdc21). Three putative donor strains were from Georgia (cdc8, cdc9, and cdc11); all other putative donor and recipient strains were collected in the Northeast. All vaccine escape strains were recovered from 2003 to 2005. Strains from 2003 to 2004 were recovered from children ≤4 y of age, although only pediatric isolates were genotyped [15]; 2005 strains were recovered from children and adults.

Sequence Homology within Regions Flanking the Capsular Locus

Regions upstream and downstream of the capsular locus were sequenced in all 51 pneumococci (Figure 1). Sequence alignment comparisons showed that recipient, donor, and progeny strains could be grouped based upon the relatedness of the regions flanking the capsular locus, considered as one joined sequence of 20.6 kb (Figure 1). The capsular locus has not yet been sequenced, but serotypes have been confirmed phenotypically, and the serotypes of a subset of the vaccine escape strains were confirmed by a PCR assay [32]. The capsular locus is considered here only as it encodes either a serotype 4 or 19A capsule.

Progeny strains consisted of three groups, P_1, P_2, and P_3 (Table 1). Twenty-seven ST695[19A] progeny strains (P_1) were identical to each other in the capsular locus flanking regions and were the major group of vaccine escape strains. There were five additional P_1 variants, all of which differed from the P_1 strains by only one to two single nucleotide polymorphisms (SNPs) over the entire region of sequence. Furthermore, one strain, P_{1var6}, was a single-locus MLST variant of the P_1 strains at *spi*, but the capsular locus flanking regions of P_1 and P_{1var6} were identical. P_2 progeny strains and strain P_3 were 0.3% and 1%, respectively, divergent from P_1 progeny over the entire region of sequence.

Among recipient strains, the flanking sequences of cdc1, cdc3, and cdc4 (R_1) were identical; recipient strain cdc7 (R_2) was 0.5% divergent from R_1 strains (Table 1). Among 19A donor strains there were four unique sequences: cdc2 and cdc8 (D_1), cdc9 (D_2), cdc11 (D_3), and cdc6 (D_4).

Recombinational Crossover Points and Likely Serotype 19A Donors among Progeny Strains

Sequence alignments of the capsular locus flanking regions revealed the upstream and downstream recombinational crossover points in the ST695[19A] strains. Three SNPs (heterologous to donor and homologous to recipient) marked and confirmed the upstream crossover point, just upstream of *pbp2x*; similarly, two SNPs marked and confirmed the down-

Table 1. Description of the Pneumococcal Strains Characterised in This Study

Strain	Strain Type	Group	Year	Sero-type	ST	MLST Allelic Profile							Source	Age (y)	State[a]	Alleles		MIC (µg/ml)[a]						
						aroE	gdh	gki	recP	spi	xpt	ddl				pbp2x	pbp1a	pen	ery	clin	cot	tet	chlo	lev
cdc1	Recipient	R[1]	1999	4	695	16	13	4	4	6	113	18	Blood	4	CT	4	1	0.03	0.06	0.12	0.25	1	4	2
cdc3	Recipient	R[1]	1999	4	695	16	13	4	4	6	113	18	Blood	2	NY	4	1	0.03	0.06	0.12	0.25	1	4	1
cdc4	Recipient	R[1]	1999	4	695	16	13	4	4	6	113	18	Blood	1	CT	4	1	0.03	0.12	0.25	0.25	1	4	1
cdc7	Recipient	R[2]	2002	4	899	16	13	4	4	6	10	18	Blood	73	MD	2	1	0.03	0.12	0.06	0.25	2	8	16
cdc2	Donor	D[1]	1999	19A	199	8	13	14	4	17	4	14	Blood	1	NY	5	5[h]	0.12	16	0.12	8	1	4	1
cdc8	Donor	D[1]	2003	19A	199	8	13	14	4	17	4	14	Blood	3	GA	5	5	0.12	32[i]	0.12	8	2	2	1
cdc9	Donor	D[2][b]	2003	19A	199	8	13	14	4	17	4	14	Blood	1	GA	6	4	0.12	32[i]	0.25	4	2	2	1
cdc11	Donor	D[3][b]	2003	19A	199	8	13	14	4	17	4	14	Blood	1	GA	5	2	0.12	0.06	0.12	0.25	2	2	1
cdc6	Donor	D[4]	1999	19A	645	7	13	14	4	17	4	14	Blood	95	CT	5	2	0.12	16	1	0.25	1	4	1
cdc10	Progeny	P[1][c]	2003	19A	695	16	13	4	4	6	113	18	Blood	<1	CT	5	5rec[h]	0.06	0.06	0.12	0.25	2	4	1
cdc13	Progeny	P[1]	2003	19A	695	16	13	4	4	6	113	18	Blood	4	NY	5	5rec	0.06	0.06	0.06	0.25	4	4	1
cdc14	Progeny	P[1]	2004	19A	695	16	13	4	4	6	113	18	Blood	3	CT	5	5rec	0.06	16[i]	0.12	0.25	2	4	1
cdc16	Progeny	P[1]	2004	19A	695	16	13	4	4	6	113	18	Blood	<1	NY	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc18	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	60	CT	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc19	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	73	NY	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc20	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	84	NY	5	5rec	0.06	0.06	0.06	0.25	2	4	1
cdc23	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	86	MD	5	5rec	0.06	0.06	0.06	0.25	2	4	1
cdc27	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	4	MN	5	5rec	0.06	0.03	0.12	0.25	2	4	1
cdc28	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	54	CT	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc29	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	4	NY	5	5rec	0.06	0.06	0.06	0.25	2	4	1
cdc30	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	68	CT	5	5rec	0.06	0.06	0.06	0.25	2	4	1
cdc31	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	79	NY	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc32	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	3	NY	5	5rec	0.12	0.06	0.06	0.25	2	4	1
cdc33	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	64	CT	5	5rec	0.06	16[i]	0.12	0.25	2	4	1
cdc36	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	67	CT	5	5rec	0.12	0.06	0.06	0.25	2	4	1
cdc38	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	87	MD	5	5rec	0.12	0.06	0.06	0.25	2	4	1
cdc39	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	4	MD	5	5rec	0.12	16[i]	0.06	0.25	2	4	1
cdc40	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	63	NY	5	5rec	0.12	0.06	0.06	0.25	2	4	1
cdc45	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	90	NY	5	5rec	0.06	0.06	0.12	0.5	2	4	1
cdc46	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	CSF[a]	42	NY	5	5rec	0.06	0.06	0.12	0.5	2	4	1
cdc47	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	66	CT	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc48	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	69	CT	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc50	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	96	CT	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc51	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	7	CT	5	5rec	0.06	0.06	0.12	0.5	2	4	1
cdc52	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	77	CT	5	5rec	0.12	0.06	0.12	0.25	2	4	1
cdc53	Progeny	P[1]	2005	19A	695	16	13	4	4	6	113	18	Blood	30	CT	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc12	Progeny	P[1var1][c]	2003	19A	695	16	13	4	4	6	113	18	Blood	3	NY	5	5rec	0.12	0.06	0.12	0.25	2	4	1
cdc17	Progeny	P[1var1]	2005	19A	695	16	13	4	4	6	113	18	Blood	1	CT	5	5rec	0.06	0.06	0.06	0.25	2	4	1
cdc22	Progeny	P[1var1]	2005	19A	695	16	13	4	4	6	113	18	Blood	9	NY	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc43	Progeny	P[1var1]	2005	19A	695	16	13	4	4	6	113	18	Blood	41	NY	5	5rec	0.12	0.06	0.12	0.25	2	4	1
cdc42	Progeny	P[1var2][c]	2005	19A	695	16	13	4	4	6	113	18	Blood	78	NY	5	5rec	0.06	0.06	0.12	0.25	2	4	1
cdc44	Progeny	P[1var2]	2005	19A	695	16	13	4	4	6	113	18	Blood	3	NY	5	5rec	0.12	0.06	0.12	0.25	2	8	1
cdc24	Progeny	P[1var3][c]	2005	19A	695	16	13	4	4	6	113	18	Blood	8	CT	7[g]	5rec	0.06	0.06	0.06	0.25	2	4	1
cdc34	Progeny	P[1var4][c]	2005	19A	695	16	13	4	4	6	113	18	Blood	40	CT	5	5rec	0.06	16[i]	0.06	0.25	2	4	1
cdc49	Progeny	P[1var5][c]	2005	19A	695	16	13	4	4	6	113	18	Blood	91	CT	5	5rec	0.12	0.06	0.12	0.25	2	4	1
cdc37	Progeny	P[1var6][d]	2005	19A	2363	16	13	4	4	136	113	18	Blood	49	CT	5	5rec	0.06	0.06	0.12	1	2	4	1
cdc25	Progeny	P[2][e]	2005	19A	2365	16	13	4	5	6	197	14	Blood	79	NY	5	2	0.06	0.06	0.25	0.25	2	4	1
cdc26	Progeny	P[2]	2005	19A	2365	16	13	4	5	6	197	14	Blood	42	NY	5	2	0.06	0.06	0.12	0.25	2	4	1
cdc35	Progeny	P[2]	2005	19A	2365	16	13	4	5	6	197	14	Blood	40	CT	5	2	0.06	8[i]	0.25	0.25	2	4	1
cdc41	Progeny	P[2var1][e]	2005	19A	2365	16	13	4	5	6	197	14	Blood	29	NY	5	2	0.06	0.06	0.06	0.25	2	4	1
cdc21	Progeny	P[3][f]	2005	19A	899	16	13	4	4	6	10	18	Blood	22	CO	3	3	0.03	0.06	0.12	0.25	2	4	1

[a]Abbreviations: CSF = cerebrospinal fluid; CO = Colorado; CT = Connecticut; GA = Georgia; MD = Maryland; MN = Minnesota; NY = New York; MIC = minimum inhibitory concentration; pen = penicillin; ery = erythromycin; clin = clindamycin; cot = cotrimoxazole; tet = tetracycline; chlo = chloramphenicol; lev = levofloxacin.

[b]Donors D[2] and D[3] were not found among this collection of progeny.

[c]P[1], P[1var1], P[1var2], P[1var3], P[1var4], and P[1var5] were the progeny of recipient R[1] and donor D[1].

[d]P[1var6] was the progeny of the recipient R[1] and donor D[1], with further evolution at the *spi* locus.

[e]P[2] and P[2var1] were the progeny of D[4] and an unknown recipient.

[f]P[3] was the progeny of recipient R[2] and an unknown donor.

[g]*pbp2x* alleles 5 and 7 differ only by a single nucleotide polymorphism.

[h]Alleles 5 and 5rec are identical except for a single nucleotide polymorphism, which marks the downstream crossover point.
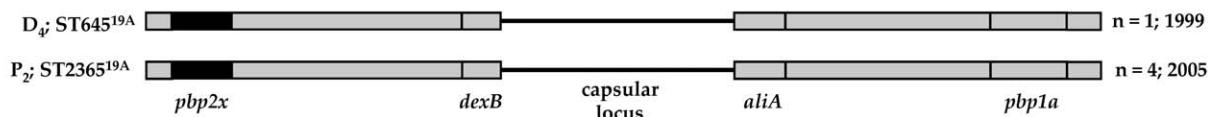
[i]Strains are efflux *mef*(A)-positive by PCR; cdc2 and cdc6 have not been tested.

doi:10.1371/journal.ppat.0030168.t001

A
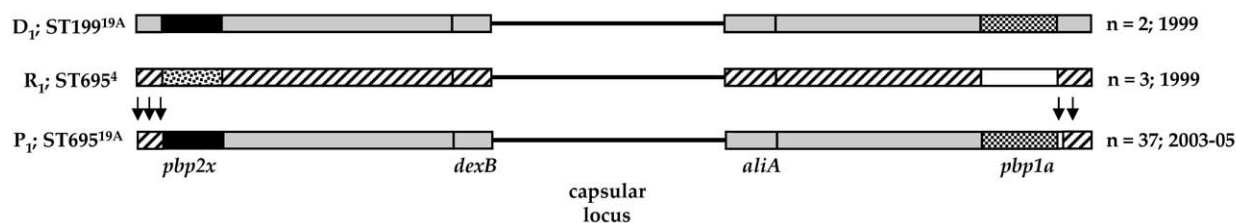
B

Figure 2. Sequencing Results among Progeny, Donor, and Recipient Pneumococci, including Differences in *pbp2x* and *pbp1a* Genes

P_1 and $P_{1variants}$, and the P_2 and $P_{2variant}$, are each represented by one P_1 and P_2 schematic, respectively, for simplicity. Unique regions of sequence are indicated by variations of shading and patterns, e.g. D_1, P_1, D_4, P_2 all have the same *pbp2x* sequence (black), whereas R_1 has a different *pbp2x* sequence (speckled).

(A) Schematic of the recombinational crossover points revealed in the major group of progeny strains, ST695[19A]. Three SNPs marked and confirmed the upstream crossover region and two SNPs marked and confirmed the downstream crossover region in the progeny strains, as shown with black arrows. The length of the recombinational fragment was 38.6 kb (10.3 kb upstream + 18.6 kb capsular locus + 9.7 kb downstream).

(B) Illustration of the second recombinational event. ST2365[19A] is identical to ST645[19A] in the entire sequenced region; no obvious crossover points have yet been revealed and the recipient is unknown.

doi:10.1371/journal.ppat.0030168.g002

stream crossover point, at the 3′ end and just beyond the 3′ end of *pbp1a* (Figures 2A and S1), resulting in a recombinational fragment of 38.6 kb. To our knowledge, this is the first reported in vivo example of a recombinational event in pneumococcus in which the capsular locus and both adjacent PBPs recombined in what appeared to be a single event. This was shown to be experimentally possible by Trzcinski and colleagues [33].

The likely donor of the serotype 19A capsular locus and flanking regions in ST695[19A] was ST199[19A]. Although the actual strain that donated the DNA cannot be identified with certainty, it is represented by the pre- and post-vaccination D_1 strains from 1999 and 2003, respectively (Figures 2A and S1).

Sequence alignments also revealed a second recombinational event around the capsular locus in four strains of P_2 progeny, all of which were first identified in 2005. Three P_2 strains were identical (or one SNP different, P_{2var1}) to D_4 in the entire flanking region sequenced (Figures 2B and S1), suggesting that D_4 was the likely donor of the 19A capsule among P_2 progeny strains.

The third type of 19A progeny strain identified in this study was P_3 (ST899[19A]). ST899 appears to have circulated at least since 1999 as a serotype 4 strain [14], but in 2005 appeared as a 19A serotype strain (Table 1). None of the sequenced 19A donors in this study share distinctive regions of homology to the P_3 progeny; thus, the donor of this 19A capsular locus is still unknown.

### Relatedness Based on MLST Genotype

Figure 3 further clarifies the evolution of these vaccine escape strains by depicting the relatedness of the background MLST genotypes. The P_1 progeny (including P_1 variants) are all in the cluster with ST695 as the founder genotype. ST695

is a single-locus variant of ST899, differing only at *xpt* (allele 113) as a result of a 39-codon deletion in the middle of the *xpt* gene. This truncated *xpt* allele was first identified in 1999 among US strains [31] and was present in ST2363[19A] and ST2284[4]. ST2363 and ST2284 differ from ST695 at *spi* and *recP*, respectively. The truncated *xpt* allele is identical to *xpt* allele 10 apart from the large deletion, thus allele 113 probably derived from the full-length allele 10 (Table 1; http://www.mlst.net/).

The P_2 progeny (including the $P_{2variant}$) appeared to evolve from the founder ST247[4] clonal complex. The oldest known strains of ST247[4] were identified in The Netherlands in 1978, and ST247[4] and the related single-locus variants in the ST247[4] cluster have been detected in other European and South American countries through at least 2005 (Figure 3; http://www.mlst.net/). The P_2 progeny were first identified in 2005 in the US. ST247[4] strains have not yet been characterised, thus recombinational crossover points in P_2 progeny have not yet been identified. The third serotype 19A capsular change was the P_3 progeny (ST899[19A]); Figure 3 depicts the relatedness of this ST to other STs in the clonal complex.

Sequence Diversity among PBPs

Alterations in PBP genes *pbp2x*, *pbp1a*, and *pbp2b* confer penicillin resistance [24–26]. Allele assignments were given to each unique *pbp2x* and *pbp1a* sequence among this collection of strains; the *pbp2x* and *pbp1a* sequences from TIGR4, a serotype 4 penicillin-susceptible strain, were used for comparison and designated allele 1 (Table 1; Figure 4). All R_1, R_2, and P_3 strains possessed *pbp2x* and *pbp1a* alleles similar to those in TIGR4, which correspond to the basal penicillin minimum inhibitory concentration (MIC) of 0.03 μg/ml that is typical of all ST695[4] isolates. Among D_3, D_4, and P_2 strains, *pbp2x* was altered while *pbp1a* was largely conserved; both
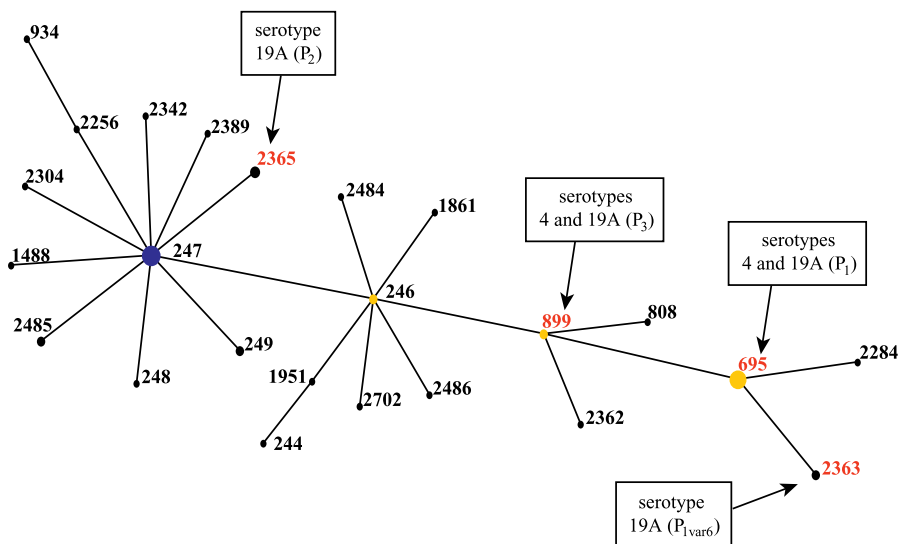
Figure 3. eBURST Analysis of Closely Related STs Found in This Study and in the MLST Database (http://www.mlst.net/) as of March 2007

STs described in the current study are indicated with arrows and boxes. The size of each circle is proportional to the number of isolates of that ST, the blue circle indicates the founder, ST247, of the entire clonal complex, and the yellow circles indicate subgroup founders (STs that have at least two single-locus variants). Strains from the ST247 founder complex have been recovered throughout Europe, South America, and the US (ST2365 recovered only in the US), from 1978 to 2005. The allelic profile of ST247 is *aroE* 16, *gdh* 13, *gki* 4, *recP* 5, *spi* 6, *xpt* 10, and *ddl* 14. Strains from the ST246 subgroup have been detected in Spain, Australia, and the United Kingdom from 1997 to 2005; strains from the ST899 subgroup have been recovered in the US and the Czech Republic from 1999 to 2002; and strains of the ST695 subgroup have been detected in the US and South Africa from 1999 to 2005. Pneumococcal strains of all STs in this diagram are serotype 4 except where indicated; however, the serotype 19A strains have only been reported in the US to date, and only since 2003. P_1, P_{1var6}, P_2, and P_3 refer to the type of progeny strains (see Table 1).
doi:10.1371/journal.ppat.0030168.g003

pbp2x and *pbp1a* were altered in the D_1, D_2, P_1, and $P_{1variant}$ strains. Phenotypically, strains with altered *pbp2x* and *pbp1a* genes resulted in a MIC to penicillin of 0.06–0.12 µg/ml (Table 1). The breakpoints for penicillin resistance are ≤0.06 µg/ml (susceptible), 0.12–1.0 µg/ml (intermediate resistance), and ≥2 µg/ml (high-level resistance), but the variation in susceptibility testing is ±1 doubling dilution [34]. All donor strains and all progeny strains apart from the P_3 strain had MIC values at the penicillin-susceptible breakpoint; however, based on the *pbp2x* and *pbp1a* sequences, D_1, D_2, P_1, and $P_{1variants}$ had divergent PBPs typical of penicillin-nonsusceptible strains ([24–26]; Figure 4). All *pbp* sequences in this study were unique to the National Center for Biotechnology Information database.

Resistance to Other Antimicrobials

Among all strains, four donor and five vaccine escape strains were erythromycin resistant, possessing the *mef*(A) efflux phenotype [29,35]; all five vaccine escape strains and two donors (cdc8 and cdc9) were confirmed to be *mef*(A)-positive by PCR ([36,37]; Lesley McGee, Emory University, personal communication). The other donor strains were not tested by PCR. One erythromycin-resistant donor strain (D_4) was also clindamycin resistant (Table 1). D_1 and D_2 donor strains were co-trimoxazole resistant, and one vaccine escape strain (cdc13) was resistant to tetracycline. One P_{1var2} strain (cdc44) was resistant to chloramphenicol; R_2 was resistant to chloramphenicol and levofloxacin.

Discussion

To our knowledge, this is the first report of vaccine escape events in the US subsequent to national pneumococcal vaccination. The main event resulted in nonvaccine serotype, penicillin-nonsusceptible ST695[19A] pneumococci as a result of recombination between the recipient ST695[4] and donor ST199[19A] (D_1). A second event resulted in the emergence of ST2365[19A] from the well-established serotype 4 clonal complex of ST247, and these strains had a different serotype 19A donor, ST645[19A]. No recipient has yet been identified, but recipient strains of ST247 will be characterised to elucidate the recombinational crossover points in these progeny. Finally, a third event resulted in the emergence of ST899[19A], but the serotype 19A donor is not yet known.

The most plausible explanation for the emergence of these strains is that one main recombinational event, ST695[19A], occurred around 2003, and the fact that it was a nonvaccine serotype variant provided the selective advantage by which these strains could evade the immune pressures invoked by the vaccine. Furthermore, with the additional selective advantage of penicillin nonsusceptibility, this progeny strain increased in frequency over the next 2 y, disseminating through the northeastern US to become the fourth most common serotype 19A genetic complex in the US (ABC surveillance program, unpublished data). The recombinational event did not appear to result in a decrease in fitness among these strains, as strains increased over time within the population. More worryingly, all of these strains were recovered from patients with bacteremia and meningitis. We cannot be absolutely certain that ST695[19A] strains never existed pre-vaccination, but extensive surveillance pre- and post-vaccination in the US failed to reveal any such strains [14,31], and no such strains have been reported to the MLST database from other parts of the world. Hence, even if these strains did exist pre-vaccination they were likely to be very rare, and it could still be maintained that the immune

```
                                                                        1 1111111111 1111111111 1111111111 1111111111 1111111122 222222222
                         112222455 5667777777 8888888999 9999999990 0000000000 0000000001 1111111111 1112233366 6678889900 000111112
                         3156777601 9022356678 5778999000 1134555580 0111222335 5677778890 0013444555 6775647939 9917788846 789126770
                         6507369574 7676511616 8068147369 2512147872 8124038290 9523473981 6737369024 1394647833 8905610391 854481235
pbp2X_allele1  CAAGACCAAG TGATAGGACT AATTAAGTGA AGGACTAGCG TCATAGTGGT TAGTCTTGTA CCTATTCAAC TTATACGAGT CGCGGACCCT CGTACCCTC
pbp2x_allele2  .......... .......... .......... .......... .......... .......... .......... .......... ...A.G..T. .........
pbp2x_allele3  .......... CA........ .......... .......... .......... .......... .......... .........T ....T....G .....G.... .A..T....
pbp2x_allele4  .......... C......... .......... .......... .......... .......... .......... .........T ...TA..G ...A.G..T. TA.....T..
pbp2x_allele5  GGGAGATCGA A.CACAAGTC TCCAGTTCTT CCATACTATA GAGCGTCATC CTTATCCAAG TGCTCCTGTT CATCT..C.. TATATGTTTG .ACCTG.AT
pbp2x_allele6  GGGAGATCGA A.CACAAGTC TCCAGTTCTT CCATACT.TA GAGCGTCATC CTTATCCAAG TGCTCCTGTT CATCT..C.. TATATGTTTG .ACCTG.AT
pbp2x_allele7  GGGAGATCGA A.CACAAGTC TCCAGTTCTT CCATACTATA GAGCGTCATC CTTATCCAAG TGCTCCTGTT CATCT..CT. TATATGTTTG .ACCTG.AT
```

```
                                                                        11 111111112
                         2233333444 4444444444 4444444444 5555555555 5555555577 8889999900 022346770
                         6813899000 1122333333 4455668899 0011122223 3344445848 0250445601 789842580
                         5966747367 2414013458 0801391469 2534734562 3812340986 5937388152 598825149
pbp1a_allele1  TGGTAACGAG TGATAATTGA GGGGCTGTAC AGCAAATTTA GAAGTCTCTT TGAGATGGCC TTGTGCAGA
pbp1a_allele2  .......... .......... .......... .A........ .......T.. G......A.. ...C...A.
pbp1a_allele3  CA........ .......... .......... .......... .......... G...G...T. ...C...A.
pbp1a_allele4  C.AC...... ..TAGTACCT TAACTAAATT TAGTGTCCAG AGTACTA.CG GA.....A.. ...C...A.
pbp1a_allele5  ..ACCGGCTA CTTAGTACCT TAACTAAATT TAGTGTCCAG AGTACTA.CG GACAGCA..T CCT.AGG.G
pbp1a_allele5rec  ..ACCGGCTA CTTAGTACCT TAACTAAATT TAGTGTCCAG AGTACTA.CG GACAGCA..T CCT.AGG..
```

Figure 4. Sequence Alignments of *pbp2x* and *pbp1a* (2,253 and 2,160 Base Pairs, Respectively, in Total Length), Showing the Polymorphic Sites (Numbered Vertically) in Each of the PBP Alleles Present in This Dataset

Note that *pbp1a* is reverse complemented. Allele 1 for each gene corresponds to the penicillin-susceptible TIGR4 pneumococcal strain [38]. *Pbp1a*_allele 5 and *pbp1a*_allele 5rec are identical apart from base pairs 1784 and 2009, which mark the downstream recombinational crossover point in progeny strains ST695[19A].

doi:10.1371/journal.ppat.0030168.g004

pressure resulting from PCV7 use selected for the emergence of such strains.

The two additional progeny strain types, ST2365[19A] and ST899[19A], were identified in 2005, and this may be the very early detection of these new recombinants. Whether or not these strains will increase in prevalence remains to be seen. Since serotype 19A was the major replacement disease serotype to emerge post-vaccination [15,17], our efforts to understand the genetics of this event have been focused on serotype 19A strains. This will be expanded to include other putative capsular switching events to better understand the phenomenon of capsular switching. Although it has been known for many years that capsular switching can and does occur, it is not at all clear how often it occurs within the pneumococcal population.

An exciting component of the current study is that recombination in the pneumococcus has been revealed almost as it occurred. This presents a unique opportunity to measure recombination *in nature* as a result of vaccine-induced changes, and may shed light on how much recombination occurs in general within the pneumococcal genome. Studies to explore these strains in detail are ongoing.

What are the implications for vaccine escape? Clearly, vaccine escape by recombination at the capsular locus has the very real potential to reduce PCV7 effectiveness in the longer term. This will almost certainly be true for any serotype-specific pneumococcal vaccine, given the diversity and complexity of serotypes possessed by the pneumococcus. These US data reinforce two key points: i) the importance of surveillance pre- and post-vaccination in countries preparing to implement PCV7, to detect changes as and when they occur; and ii) the importance of understanding the genetic events that result in vaccine escape. Discerning the genetic event is crucial to understanding vaccine escape and pneumococcal recombination in general. Such knowledge will provide guidance about the design and use of future pneumococcal vaccines.

Materials and Methods

Invasive pneumococcal strains were collected as part of the ABC surveillance program at the CDC. Microbiological testing and molecular characterisation by MLST was performed as previously described [14,31]. Strains for this study were selected from pre- and post-vaccine implementation ABC collections and sent to the University of Oxford. Sequence alignments of the TIGR4 [38], R6 [39], and Spain[23F-1] (http://www.sanger.ac.uk/Projects/S__pneumoniae/) pneumococcal genomes were used to design 51 sets of PCR primers (available upon request) in regions upstream and downstream of the capsular locus (Figure 1).

DNA was extracted from pneumococcal strains using the DNeasy Tissue Kit (Qiagen UK). PCR assays were identical for all combinations of PCR primers: 2.5 µl of PCR buffer (Qiagen), 0.5 µl of dNTPs (200 µM stock), 0.5 µl of forward primer (100 µM stock), 0.5 µl of reverse primer (100 µM stock), 0.3 µl of Taq polymerase (Qiagen UK), 19.7 µl of nuclease-free water (Invitrogen UK), and 1 µl (~1 µg) of extracted DNA. Thirty-five cycles of PCR amplification were performed: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 60 s. Ten-microliter sequencing reactions were prepared with 4 µl primer (1 µM stock of PCR primers), 1.75 µl sequencing buffer (Applied Biosystems), 0.5 µl Big Dye (Applied Biosystems), 1.75 µl nuclease-free water, and 2 µl cleaned PCR amplicons; followed by 25 sequencing cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 2 min. PCR amplicons and sequencing products were cleaned as described previously [15].

Sequences were assembled and edited using Pregap4 and Gap4 (Staden Package, http://staden.sourceforge.net/). Consensus sequences were aligned using Multalin [40] and trimmed to the longest possible consensus sequence shared by all strains. Trimmed sequences were uploaded into MEGA version 3.1 [41], and compared in an iterative process to ascertain which strains were donors of the 19A capsule and reveal recombinational crossover points in progeny strains. The eBURST algorithm was used to compare all progeny strains to all pneumococcal strains represented in the MLST database [42].

Supporting Information

Figure S1. Sequence Alignments of Major Donor, Recipient, and Progeny Strains in This Study, Aligned with the Corresponding Region of the TIGR4 Sequence

Polymorphic sites are shown across the entire sequenced region upstream and downstream of the capsular locus. Black arrows highlight the SNPs in the upstream and downstream crossover regions; the locations of *pbp2x*, *pbp1a*, and the (missing) capsular locus are also shown. Strains are labeled A and B to correspond to the strains involved in the two main recombinational events shown in Figure 2.

Found at doi:10.1371/journal.ppat.0030168.sg001 (95 KB PPT).

References

1. World Health Organization (2007) Pneumococcal conjugate vaccine for childhood immunization–WHO position paper. Wkly Epidemiol Rec 82: 93–104.
2. Black S, Shinefield H, Fireman B, Lewis E, Ray P, et al. (2000) Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Pediatr Infect Dis J 19: 187–195.
3. O'Brien KL, Moulton LH, Reid R, Weatherholtz R, Oski J, et al. (2003) Efficacy and safety of seven-valent conjugate pneumococcal vaccine in American Indian children: group randomised trial. Lancet 362: 355–361.
4. Whitney CG, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. (2003) Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. N Engl J Med 348: 1737–1746.
5. Lexau CA, Lynfield R, Danila R, Pilishvili T, Facklam R, et al. (2005) Changing epidemiology of invasive pneumococcal disease among older adults in the era of pediatric pneumococcal conjugate vaccine. JAMA 294: 2043–2051.
6. Whitney CG, Pilishvili T, Farley MM, Schaffner W, Craig AS, et al. (2006) Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. Lancet 368: 1495–1502.
7. Bentley SD, Aanensen DM, Mavroidi A, Saunders D, Rabbinowitsch E, et al. (2006) Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet 2: e31. doi:10.1371/journal.pgen.0020031
8. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, et al. (2007) Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*. J Clin Microbiol 45: 1225–1233.
9. Crook DW, Brueggemann AB, Sleeman K, Peto TEA (2004) Pneumococcal carriage. In: Tuomanen EI, Mitchell TJ, Morrison DA, Spratt BG, editors. The pneumococcus. Washington (D.C.): ASM Press. pp. 136–147.
10. Butler JC (2004) Epidemiology of pneumococcal disease. In: Tuomanen EI, Mitchell TJ, Morrison DA, Spratt BG, editors. The pneumococcus. Washington (D.C.): ASM Press. pp. 148–168.
11. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, et al. (2003) Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential. J Infect Dis 187: 1424–1432.
12. Brueggemann AB, Peto TE, Crook DW, Butler JC, Kristinsson KG, et al. (2004) Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. J Infect Dis 190: 1203–1211.
13. Enright MC, Spratt BG (1998) A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiology 144: 3049–3060.
14. Beall B, McEllistrem MC, Gertz RE, Wedel S, Boxrud DJ, et al. (2006) Pre- and postvaccination clonal compositions of invasive pneumococcal serotypes for isolates collected in the United States in 1999, 2001 and 2002. J Clin Microbiol 44: 999–1017.
15. Pai R, Moore MR, Pilishvili T, Gertz RE, Whitney CG, et al. (2005) Postvaccine genetic structure of *Streptococcus pneumoniae* serotype 19A from children in the United States. J Infect Dis 192: 1988–1995.
16. Byington CL, Samore MH, Stoddard GJ, Barlow S, Daly J, et al. (2005) Temporal trends of invasive disease due to *Streptococcus pneumoniae* among children in the intermountain west: emergence of nonvaccine serogroups. Clin Infect Dis 41: 21–29.
17. Hicks LA, Harrison LH, Flannery B, Hadler JL, Schaffner W, et al. (2007) Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998–2004. J Infect Dis 196: 1346–54.
18. Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, et al. (2007) Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska Native children with high levels of 7-valent pneumococcal vaccine coverage. JAMA 297: 1784–1792.
19. Coffey TJ, Dowson CG, Daniels M, Zhou J, Martin C, et al. (1991) Horizontal transfer of multiple penicillin-binding protein genes, and capsular biosynthetic genes, in natural populations of *Streptococcus pneumoniae*. Mol Microbiol 5: 2255–2260.
20. Coffey TJ, Enright MC, Daniels M, Morona JK, Morona R, et al. (1998) Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of *Streptococcus pneumoniae*. Mol Microbiol 27: 73–83.
21. Coffey TJ, Daniels M, Enright MC, Spratt BG (1999) Serotype 14 variants of the Spanish penicillin-resistant serotype 9V clone of *Streptococcus pneumoniae* arose by large recombinational replacements of the *cpsA-pbp1a* region. Microbiology 145: 2023–2031.
22. Spratt BG, Greenwood BM (2000) Prevention of pneumococcal disease by vaccination: does serotype replacement matter? Lancet 356: 1210–1211.
23. Llull D, Muñoz R, López R, García E (1999) A single gene (*tts*) located outside the cap locus directs the formation of *Streptococcus pneumoniae* type 37 capsular polysaccharide. Type 37 pneumococci are natural, genetically binary strains. J Exp Med 190: 241–251.
24. Coffey TJ, Daniels M, McDougal LK, Dowson CG, Tenover FC, et al. (1995) Genetic analysis of clinical isolates of *Streptococcus pneumoniae* with high-level resistance to expanded-spectrum cephalosporins. Antimicrob Agents Chemother 39: 1306–1313.
25. Grebe T, Hakenbeck R (1996) Penicillin-binding proteins 2b and 2x of *Streptococcus pneumoniae* are primary resistance determinants for different classes of β-lactam antibiotics. Antimicrob Agents Chemother 40: 829–834.
26. Muñoz R, Dowson CG, Daniels M, Coffey TJ, Martin C, et al. (1992) Genetics of resistance to third-generation cephalosporins in clinical isolates of *Streptococcus pneumoniae*. Mol Microbiol 6: 2461–2465.
27. Hoban D, Baquero F, Reed V, Felmingham D (2005) Demographic analysis of antimicrobial resistance among *Streptococcus pneumoniae*: worldwide results from PROTEKT 1999–2000. Int J Infect Dis 9: 262–273.
28. Kyaw MH, Lynfield R, Schaffner W, Craig AS, Hadler J, et al. (2006) Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant *Streptococcus pneumoniae*. N Engl J Med 354: 1455–1463.
29. Farrell DJ, Klugman KP, Pichichero M (2007) Increased antimicrobial resistance among nonvaccine serotypes of *Streptococcus pneumoniae* in the pediatric population after the introduction of 7-valent pneumococcal vaccine in the United States. Pediatr Infect Dis J 26: 123–128.
30. Schuchat A, Hilger T, Zell E, Farley MM, Reingold A, et al. (2001) Active Bacterial Core surveillance of the Emerging Infections Program network. Emerg Infect Dis 7: 92–99.
31. Gertz RE, McEllistrem MC, Boxrud DJ, Li Z, Sakota V, et al. (2003) 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 41: 4194–4216.
32. Pai R, Gertz RE, Beall B (2006) Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates. J Clin Microbiol 44: 124–131.
33. Trzcinski K, Thompson CM, Lipsitch M (2004) Single-step capsular transformation and acquisition of penicillin resistance in *Streptococcus pneumoniae*. J Bacteriol 186: 3447–3452.
34. Clinical and Laboratory Standards Institute (2007) Performance standards for antimicrobial susceptibility testing. 17th informational supplement, CSLI document M100-S17E. Wayne (Pennsylvania): Clinical and Laboratory Standards Institute. pp. 64–68.
35. Brueggemann AB (2006) Antibiotic resistance mechanisms among pediatric respiratory and enteric pathogens: a current update. Pediatr Infect Dis J 25: 969–973.
36. Sutcliffe J, Grebe T, Tait-Kamradt A, Wondrack L (1996) Detection of erythromycin-resistant determinants by PCR. Antimicrob Agents Chemother 40: 2562–2566.
37. Widdowson C, Klugman KP (1998) Emergence of the M-phenotype of erythromycin-resistant pneumococci in South Africa. Emerg Infect Dis 4: 277–281.
38. Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, et al. (2001)

Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science 293: 498–506.

39. Hoskins J, Alborn WE, Arnold J, Blaszczak LC, Burgett S, et al. (2001) The genome of the bacterium *Streptococcus pneumoniae* strain R6. J Bacteriol 183: 5709–5717.

40. Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucl Acids Res 16: 10881–10890.

41. Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform 5: 150–163.

42. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG (2004) eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol 186: 1518–1530.