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Pneumococcal sequence type replacement among American Indian children: A comparison of pre- and routine-PCV7 eras

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ABSTRACT

Background: Multi-locus sequence typing (MLST) of pneumococcal isolates collected during an efficacy trial of the 7-valent pneumococcal conjugate vaccine (PCV7) among Navajo and White Mountain Apache children from 1998 to 2000 showed a non-differential expansion of pre-existing sequence types (STs) and only one capsule-switching event in the PCV7-randomized communities. PCV7 was introduced as a routine infant vaccine in October 2000. We assessed variability in PCV7 effectiveness and mechanisms of ST replacement after prolonged routine PCV7 use.
Methods: We applied MLST to 267 non-vaccine type pneumococcal carriage and invasive disease isolates from Navajo and White Mountain Apache children from 2006 to 2008, and compared them to those from 1998 to 2000. Microarray was used to confirm capsule switching events.
Results: The primary mechanism of ST replacement among Navajo and White Mountain Apache children was expansion of existing STs, although introduction of new STs was an important secondary mechanism. ST199, a majority being serotype 19A, was the most common ST in both eras. Only ST193 (serotype 21) was preferentially expanding in the PCV7 era. Three examples of capsule switching were identified. No variability in vaccine effectiveness by ST was observed.
Conclusion: We did not observe an influence of ST on PCV7 serotype-specific effectiveness, although some STs may be favored in replacement.

1. Background

Prevention of serious infections among children from *Streptococcus pneumoniae* (pneumococcus) is a major global health objective. The 7-valent pneumococcal polysaccharide conjugate vaccine, PCV7 (Prev(e)nar®; Pfizer, NY), contains capsular polysaccharide antigen of seven pneumococcal serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F). Routine use of PCV7 among infants in the United States (US) has made a significant impact on vaccine serotype (VT) invasive pneumococcal disease (IPD) and nasopharyngeal (NP) carriage among age groups targeted for vaccination as well as other age groups. However, studies have documented variability of PCV7 efficacy by serotype [1–3] and have documented increased rates of IPD and NP carriage with non-vaccine serotypes (NVT) following routine PCV7 use, a phenomenon termed "serotype replacement" [2,4–7].

In addition to capsular serotype, variability of pneumococcal genes at sites outside the capsule polysaccharide biosynthesis locus may play a role in observed PCV7 effectiveness against and replacement with specific serotypes. A common method used to classify the genetic background of bacteria is multi-locus sequence typing (MLST) [8,9]. MLST has been used to understand mechanisms behind PCV7 effectiveness and replacement observed in carriage and IPD [10–13]. While PCV7 targets serotypes, specific sequence types (STs) of NVTs have been identified as responsible for the majority of replacement in disease and carriage, particularly ST199 with serotype 19A. Serotype 19A is a vaccine-associated NVT, defined as a serotype within the same serogroup as a vaccine serotype.

Given the expanding use of PCV products globally, it is relevant that we understand the mechanisms behind replacement and improve our understanding of pneumococcal adaptations to

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vaccine pressure. This is particularly important for developing country settings where IPD burden is high, and where optimizing PCV impact strongly influences policy decisions for vaccine use because of limited financial resources. Because Navajo and White Mountain Apache populations, located in the southwest of the United States, have some of the highest reported rates of IPD and carriage in the world [14,15] epidemiologic effects of PCV have relevance beyond these communities, particularly for countries with similarly high pneumococcal disease burden.

Since routine PCV7 introduction among Navajo and White Mountain Apache in late 2000, NVT strains have fully replaced VT colonizing strains prevalent before introduction of PCV [16]. Although rare, VTs are still occasionally identified in carriage and IPD in these communities in the PCV7 era with a majority being serotype 19F [16,17]. Prior to routine use, during the PCV7 efficacy trial conducted among Navajo and White Mountain Apache children from 1997 to 2000, early sequence type replacement in carriage and IPD was primarily the result of an expansion of pre-existing NVT STs that already circulated in the community before introduction of PCV7 [13]. No statistically significant variability in vaccine effectiveness against STs and no preferential expansion of a particular ST were identified. Only one episode of capsule switching from a vaccine serotype to a non-vaccine serotype was observed. However vaccine effectiveness by ST and ST replacement after long-term routine use of PCV7 has not been evaluated among the Navajo and White Mountain Apache, populations with some of the longest PCV use anywhere in the world. The adaptive abilities of the pneumococcus through recombination and horizontal gene transfer enhance its ability to adapt to vaccine pressure. These adaptations could take years to occur, both because evolutionary response to selective pressure may require multiple rounds of transmission, and because selective pressure is likely increasing over time as effective vaccine coverage increases [18].

This study assessed the mechanism of pneumococcal ST replacement, changes in circulating ST diversity, and possible ST variability in PCV7 effectiveness by comparing ST compositions of isolates from periods before and after prolonged PCV7 use among Navajo and White Mountain Apache people. Based on experience from the community-randomized PCV7 trial [13], we hypothesized that PCV7 effectiveness would be serotype and not ST specific and that replacement will primarily be an expansion of pre-existing STs with some outgrowth of particular STs. Understanding the implications of vaccine effectiveness and replacement over the longer term will help predict the continuing effectiveness of PCVs of greater valency now in use, as well as anticipate the impact of PCVs on pneumococcal disease globally.

2. Methods

2.1. Study population

The study was conducted among Navajo and White Mountain Apache families living on or near the reservations located in Arizona and New Mexico. PCV7 was first introduced among those <2 years of age in 1997 as part of a community randomized efficacy trial, then used as part of the routine immunization schedule since October 2000 with catch-up immunizations for those <5 years of age. Greater than 90% coverage with 3-doses of PCV7 by 19–35 months of age was achieved and sustained by 2003 [17].

2.2. Study design

This study assessed the ST composition of pneumococcal isolates from children <5 years of age in the routine PCV7 era. NP carriage samples were from a prospective, longitudinal, observational cohort study conducted from March 2006 to March 2008 among Navajo and White Mountain Apache families. Each family was visited monthly by a trained field-worker over a six-month period (seven visits total). NP swabs were collected at each visit. A representative sample of carriage serotypes from children <5 years of age were chosen for this analysis. We used the 861 first acquisition isolates (as opposed to all carriage isolates) as the sampling frame to avoid testing multiple isolates from the same carriage episode. From these we selected 250 isolates as follows: we calculated each serotype's proportion among the 861 total, and within each serotype randomly selected isolates to represent the same proportion in a total of 250 carriage isolates. IPD isolates were from active surveillance of clinical microbiology laboratories serving these communities during the same time period [17]. Children <5 years of age who resided in the carriage cohort study communities, and who had an incident episode of IPD identified through the active surveillance system between 2006 and 2008, were chosen for this analysis.

We compared the 2006–2008 MLST data to the previously published 1998–2000 MLST data from the same population [13]. The 1998–2000 data used in the present analysis were from the control vaccine communities of the PCV7 trial to best mirror the pre-PCV7 environment. Carriage and otitis media (OM) isolates were from children <2 years of age enrolled in the PCV7 efficacy trial [19,20] and IPD isolates of children <2 years of age were identified through active surveillance of IPD during this time period [17]. The randomized efficacy trial 'communities' were defined by taking into consideration characteristics that would predict the least amount of intercommunity contact as previously described [21].

2.3. Pneumococcal isolation

NP specimens were obtained and pneumococcus isolated using methods described elsewhere [16].

2.4. Multilocus sequence typing

Sequence types of pneumococcal isolates were determined by MLST [9] and DNA samples for MLST were prepared as described elsewhere [22]. PCR products were sequenced using a Prism 3730xl Genetic Analyzer (Applied Biosystems). The raw sequences were analyzed using Molecular Evolutionary Genetics Analysis 4 software (www.megasoftware.net) and alleles/STs assigned using the MLST database (http://spneumoniae.mlst.net). The eBURST algorithm (version 3, http://eburst.mlst.net) was used to group STs into "clonal complexes" (CCs) composed of closely related STs [23]. To determine whether the ST was previously associated with a particular serotype(s), we searched the MLST database [24].

2.5. Molecular serotyping

A microarray designed for molecular serotyping of the pneumococcus was used to verify the Quellung serotype of isolates we suspected of capsule switching, based on MLST results, from vaccine serotype to non-vaccine serotype. The microarray was designed by the Bacterial Microarray Group at St. George's, University of London (BμG@S; http://bugs.sgul.ac.uk/), and manufactured on the Agilent SurePrint 8 × 15K platform (Agilent Technologies) [25]. The microarray included reporters to represent all genes involved in capsule polysaccharide biosynthesis of the 91 serotypes known to date [26,27]. DNA samples for microarray were isolated using Qiagen DNeasy Mini Spin Column kits per manufacturer's instructions. Agilent's comparative genomic hybridization protocol was used to label the DNA and hybridize the labeled sample

to the microarray slide. The microarray data were visualized using GeneSpring software (Agilent Technologies).

2.6. Analysis

Each NVT pneumococcal sequence type was classified as occurring in the pre-PCV7 era (1998–2000), routine PCV7 era (2006–2008), or both. We limited the analysis to NVTs as VTs have been nearly eliminated from the population in the routine PCV7 era [16,17]. The proportion of isolates with an ST unique to the routine PCV7 era was calculated to determine whether the mechanism of replacement was primarily due to an expansion of pre-existing STs or an introduction of new STs. To assess whether the frequency of a NVT sequence type had increased relative to all other NVT STs in the routine PCV7 era, a contingency table was constructed for each NVT. For example, the two rows in the ST199 table were the frequency of ST199 and the frequency of all other NVT STs. The columns were pre-PCV7 and routine-PCV7 eras. To determine evidence of capsule switching among the isolates, VT sequence types that occurred in both eras but expressed a NVT serotype in the routine PCV7 era were identified and serotype confirmed using microarray.

We compared differences in ST composition between pre- and routine-PCV7 era samples by applying a permutation test to a classification index as described elsewhere [28]. Sequence type diversity by era was assessed using Simpson's index of diversity (D), which is the probability that two randomly selected isolates have a different ST [29,30]. The confidence intervals were estimated using the method of Grundmann et al. [31]. The statistical analysis for the microarray was performed using a custom web-based tool developed for analysis of the BμG@S SP-CPS microarray as described elsewhere [32].

Institutional Review Boards of the Johns Hopkins Bloomberg School of Public Health, the Navajo Nation, and the Phoenix Area Indian Health Service as well as the White Mountain Apache tribes approved the studies used for these analyses. Adults and parents or guardians of children enrolled in the NP carriage cohort study provided written informed consent.

3. Results

A total of 267 carriage and IPD isolates from 2006 to 2008 were analyzed by MLST and compared to the MLST results of 297 pre-PCV7 carriage, OM, and IPD isolates from 1998 to 2000 [13]. OM samples were not analyzed in the 2006–2008 era. The average age of study subjects was 24 months in the 2006–2008 analysis and 15 months in the 1998–2000 analysis. The MLST analysis from the 1998 to 2000 efficacy trial was limited to children <2 years of age. We explored the possibility of age bias and biases due to comparisons of IPD and NP sample data to IPD, NP, and OM sample data, by repeating our analyses comparing only children <2 years of age in both eras and by comparing IPD data to IPD data and NP data to NP data. These sub-analyses resulted in the same inferences drawn from the main analyses; therefore we only report the results of the main analyses here.

Table 1 describes the proportion of isolates that were VT, NVT, and vaccine-associated types (VAT, a subset of NVT) analyzed from carriage, OM, and IPD by era. While VTs comprised the largest proportion of isolates prior to PCV7 introduction, NVTs comprised the majority of isolates found in the routine PCV7 era. The proportions that were VAT remain similar in both eras. A summary of all STs identified by era, site of isolation and serotype can be found in the Appendix. Seven isolates analyzed from the routine PCV7 era were VT. Of these, four were serotype 19F with different underlying STs. These STs are all associated with serotype 19F, except ST227, which is associated with serotype 1 [10,24]. Vaccine serotypes 4, 6B, and 9V carriage isolates that were analyzed had STs typically associated with their respective serotypes.

Table 1
Isolates analyzed by era, site of isolation, and type.

	Carriage	OM	IPD
Pre-PCV7 era[a]			
VT	64 (36.2%)	19 (32.2%)	32 (52.5%)
NVT	113 (63.8%)	40 (67.8%)	29 (47.5%)
VAT	53 (29.9%)	15 (25.4%)	10 (16.4%)
Total	177	59	61
Routine PCV7 era			
VT	6 (2.8%)	na	1 (1.9%)
NVT	208 (97.2%)	na	53 (98.1%)
VAT	57 (26.6%)	na	10 (18.5%)
Total	214	na	54

[a] Previously published data [13]

3.1. Distinct populations in pre and routine vaccine eras

The pneumococcal population snapshot in Fig. 1, was generated by the eBURST program (http://eburst.mlst.net). The figure shows the relationship between different STs and compares the isolates identified prior to and after routine PCV7 use [23]. A total of 159 STs were identified, 59 of which were unique to the 1998–2000 isolates (black) and 55 unique to the 2006–2008 isolates (green). Forty-five STs were found in both eras (pink). Twenty-five of the 2006–2008 STs were novel to the MLST database at the time of the analysis. Of the STs found among the 2006–2008 isolates, the majority (55%) were not found in the 1998–2000 isolates, suggesting that they have either been introduced into this population since routine vaccination, or were previously too rare to be detected. However, 64.0% of the 2006–2008 isolates had an ST that was found prior to PCV7 introduction, suggesting expansion of pre-existing STs was still an important mechanism of ST replacement in the routine PCV7 era.

The composition of STs was significantly different between pre- and routine-PCV7 eras as measured by the classification index (test statistic = 0.534; $p < 0.01$). However, overall ST diversity did not significantly change (Table 2). Sequence type diversity was also assessed within specific NVT serotypes (Table 3). Only serotype 19A showed a significant increase in diversity (i.e. the probability that two randomly selected 19A isolates would have a different ST) from 0.301 (95% CI: 0.119–0.483) prior to PCV7 introduction, to 0.735 (95% CI: 0.629–0.841) after routine PCV7 use. Thirty-three NVT STs were found in the two eras combined and Fisher's exact tests comparing NVT ST proportions by era showed only one of these 33 sequence types, ST193 (found only with serotype 21 in this study), expanding disproportionately compared to the other NVT STs (OR: 8.72, 95%CI: 1.27–374).

Table 4 shows the ten most common STs found among isolates from IPD and carriage by era, and includes the serotypes associated with them according to the MLST database [24]. ST199 was the most common ST found in both eras. STs associated with serotypes 15B/C, particularly ST199, and serotype 11A with ST62, continue to figure prominently in both eras. ST395, which had been associated with serotype 6A, is no longer a prominent ST in this population suggesting cross protection may have been provided by the serotype 6B component of PCV7.

Table 2
Sequence type diversity indices by era and serotype grouping.

Era	Type	Diversity index (95%CI)
PVE (1998–2000)	All types	0.978 (0.972, 0.984)
PVE (1998–2000)	VAT/NVT	0.958 (0.944, 0.972)
RVE (2006–2008)	All Types	0.982 (0.978, 0.986)
RVE (2006–2008)	VAT/NVT	0.981 (0.977, 0.985)

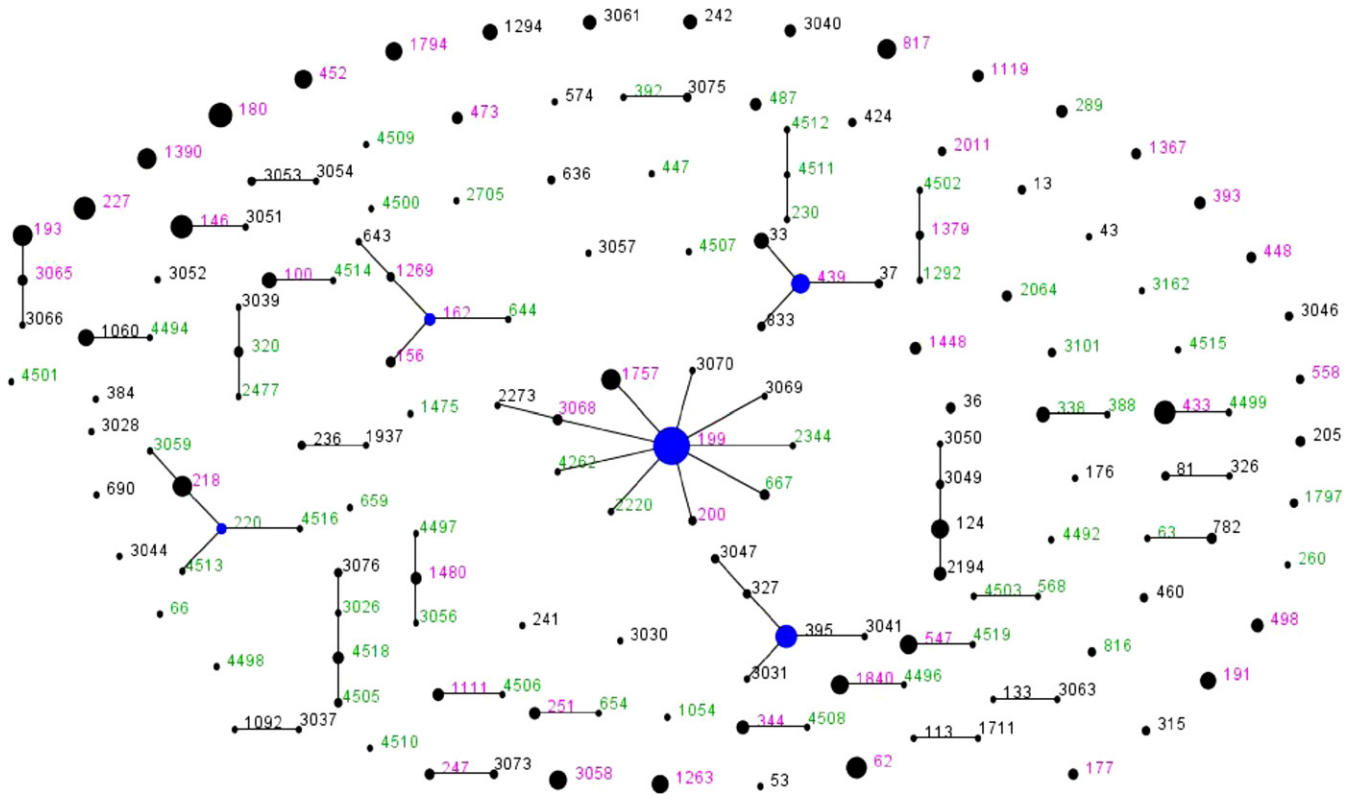

Fig. 1. eBURST diagram comparing STs found in 1998–2000 [13] and 2006–2008.

3.2. Capsule switching

We identified eight isolates as potential examples of capsule switching, where a VT sequence type was assigned to the isolate by MLST but the Quellung serotype was NVT. We further analyzed these isolates by microarray to verify the Quellung serotype using the pneumococcal TIGR4 strain as a control (Table 5). We confirmed the Quellung serotyping was correct for the eight isolates and identified three unique types of capsule switching among them. Three serotype 35B carriage isolates of ST162 and one isolate of the closely related ST644 were found in the routine PCV7 era. Prior to PCV7 introduction, in both Navajo and White Mountain Apache communities as well as other populations, this clonal complex was primarily associated with vaccine serotypes 6B, 9V, and 19F and has not been found with serotype 35B thus far [10,24]. A serotype 35B carriage isolate of ST344 was also found in the routine PCV7 era. ST344 has generally been associated with pneumococci that are

Table 3
Sequence type diversity indices by era and serotype.[a].

Serotype	PVE[b] diversity indices (95%CI)	RVE diversity indices (95%CI)
3	0.167 (−0.099–0.433)	0.556 (0.250–0.861)
7C	0	0.900 (0.725–1.08)
7F	0	0.250 (−0.101–0.601)
8	0.667 (0.304–1.03)	0.833 (0.583–1.08)
10A	0	0.429 (0.122–0.735)
11A	0.333 (−0.072–0.739)	0
12F	0	0.750 (0.615–0.885)
15A	0.571 (0.465–0.678)	0.603 (0.460–0.745)
15B	0.667 (0.304–1.03)	0.933 (0.805–1.06)
15C	0.929 (0.840–1.02)	0.900 (0.725–1.08)
16F	0	0.417 (0.047–0.786)
17F	0.667 (0.304–1.03)	0.222 (−0.104–0.548)
19A	0.301 (0.119–0.483)	0.735 (0.629–0.841)
22F	0.467 (0.235–0.699)	0.154 (−0.096–0.404)
23A	0.500 (0.067–0.933)	0.464 (0.082–0.847)
23B	0	0.576 (0.258–0.893)
31	0	0.900 (0.725–1.08)
33F	0.800 (0.657–0.943)	0.643 (0.301–0.965)
34	0	0.222 (−0.104–0.548)
35A	0	0.700 (0.349–1.05)
35B	0.400 (−0.029–0.829)	0.727 (0.523–0.931)
35F	0	0.667 (0.409–0.925)
38	0	0.667 (0.304–1.03)
NT	0.927 (0.886–0.968)	0.915 (0.864–0.965)

The yellow highlights the only serotype with statistically significant differences in diversity indices between the two eras.

[a] Excluded serotypes 6A and 6C.

[b] Prevalence data previously published [13].

Table 4
Most common STs found in carriage and IPD by era.

ST	Count	Percentage (%)	Serotypes
Pre-PCV7 era (1998–2000)			
199	27	14.84	15B, 15C, 19A, 23F
395	15	8.24	6A/6C
180	11	6.04	3
1757	8	4.40	11A, 15B, 15C, 15F, 7C
218	8	4.40	12F
1294	7	3.85	22F
227	6	3.30	1
3061	6	3.30	9L, NT
1390	5	2.75	6A/6C
3058	5	2.75	15A, 15C
Routine PCV7 era (2006–2008)			
199	16	6.13	15B, 15C, 19A, 23A, NT
193	12	4.60	15C, 21
433	12	4.60	22F
62	9	3.45	11A
1794	8	3.07	17F
227	8	3.07	1, 19F, NT
439	8	3.07	23B
547	8	3.07	34, NT
1263	7	2.68	10A, 10C
1390	7	2.68	6A, 6C

Table 5
Sequence types and serotypes of capsule switch candidates.

Isolate ID	Era	Source	Quellung serotype	ST	Serotypes associated with ST[a]	Microarray serotype
1396-07	RVE	NP	35B	162	19F, 14, and 9V	35B
2920-08	RVE	NP	35B	162	19F, 14, and 9V	35B
8543-08	RVE	NP	35B	162	19F, 14, and 9V	35B
1004-08	RVE	NP	35B	344	19F, 6A, and NTs	35B
1386-07	RVE	NP	35B	644	19F, 6B, and 9V	35B
09397	RVE	IPD	6C	2064	18C	6C
2090-07	RVE	NP	6C	2064	18C	6C
2804-06	RVE	NP	6C	2064	18C	6C

[a] Per the MLST online database [24].

nontypeable by the Quellung reaction, however the MLST database does contain two incidences of ST344 having vaccine serotypes 19F and 6A. Two serotype 6C carriage isolates and one serotype 6C IPD isolate of ST2064 were found among the 2006–2008 isolates. This ST had previously been associated with vaccine serotype 18C only.

4. Discussion

The results of this study suggest multiple mechanisms of ST replacement in the routine vaccine era among the Navajo and White Mountain Apaches. The pneumococcal population in the routine PCV7 era has arisen both by the expansion of STs already common prior to vaccine introduction (e.g. ST 199), and through the successful expansion of STs which were not found in the pre-PCV7 era, presumably because they were either too rare to be detected or because they have been introduced from elsewhere.

Given the small proportion of vaccine serotypes identified in the routine PCV7 era with no clear partiality for a particular ST within those serotypes, there does not appear to be variability in PCV7s effectiveness by ST. The heterogeneity in representation of STs in the PCV7 era suggests selective advantages for some, such as the continued high proportion of ST199 and the expansion of ST193. During the vaccine trial, ST193 with serotype 21 was more frequently found in PCV7-randomized communities as compared to control communities (7/218 NVTs vs. 1/184 NVTs), although this was not statistically significant [13]. In this dataset, ST193 is almost exclusively serotype 21 (there are two isolates with a 15C capsule) but has also been found in unvaccinated populations with vaccine serotypes 14 and 18C [24]. In other vaccinated populations, ST193 has been associated with serotype 19A [24].

The classification index result indicates that a shift in the composition of STs occurred after PCV7 introduction via the disappearance of VTs and replacement by an expansion of pre-existing and introduction of new NVTs. Additionally, while overall ST diversity between the eras did not significantly differ per Simpson's diversity index, the diversity of the NVT subset had significantly increased after routine PCV7 use. Among individual serotypes, only 19A had a statistically significant increase in ST diversity among the 2006–2008 isolates.

There are several limitations to this study. We attribute our pneumococcal population changes to PCV7 introduction, but secular changes in ST composition within a population may also occur absent vaccine. Comparisons of ST data to the MLST database are limited by its current size and scope. We used the database to identify serotypes generally associated with certain STs and to identify possible capsule switching events. However as additional contributions are made to the MLST database, previously unidentified serotype/ST associations will emerge, increasing the range of what we know to be "generally associated".

Our conclusion here that ST replacement is a combination of expansion of pre-existing STs and an introduction or identification of many new STs in this population differs from the conclusions about pneumococcal strain ecology made during the PCV7 efficacy trial among the Navajo and White Mountain Apache as well as the experience in Massachusetts [12,13]. At the outset of PCV7 use, NVT replacement was primarily driven by an expansion of pre-existing STs; more time was needed for the introduction of a large number of new STs into the population and expansion of those strains. Our diversity analysis of the 2006–2008 strains, however, are similar to findings from Massachusetts, wherein the pneumococcal population changed significantly from 2001 to 2004, but unlike our findings overall ST diversity was not significantly different since the diversity was due to expansion of existing NVT STs [12].

ST199 strains, a majority being serotype 19A, continue to be the most important ST in IPD and carriage in the routine PCV7 era among Navajo and White Mountain Apache children. We have also identified ST156 and ST320 serotype 19As among the carriage isolates. In other communities, ST156 had previously been associated with a drug-resistant serotype 9V IPD clone before introduction of PCV7 [24], but is now routinely found among serotype 19A IPD isolates in studies across the US [33–35]. ST320, a product of a capsule switch event from serotype 19F, is known to be an invasive and drug-resistant ST now associated with serotype 19A [11,35]. It has only recently emerged in the US, specifically in Massachusetts in 2005, and highlights how quickly STs can spread across a geographical area [11].

The introduction of the 13-valent pneumococcal conjugate vaccine, PCV13 (Prev(e)nar 13®; Pfizer, NY), which includes serotype 19A, will likely have a profound impact on carriage of and IPD caused by the included serotypes regardless of the underlying sequence type of the pneumococcus. While PCV7 was tailored to important serotypes in developed countries, PCV13 includes the top 5 serotypes identified in a global serogroup distribution analysis for all regions [36]. Approximately 40% of IPD and 15% of carriage serotypes from Navajo and White Mountain Apache communities between 2006 and 2008 are included in PCV13 [16,17]. However, there are serotypes that continue to be important in carriage and/or IPD among Navajo and White Mountain Apache that are not included in PCV13, including serotypes 12F (IPD and carriage), 15A (IPD), and 15B/C, 21, and 35B (carriage). The probable ST diversification observed in serotype 12F, the ST199 association with 15B/C, capsule switching of serotype 35B, and preferential expansion of serotype 21 with ST193 since introduction of PCV7 warrants continued surveillance of serotypes and sequence types in the PCV13 era.

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Potential conflicts of interest: KLO and MS have previously received grant support and/or honoraria from Wyeth Vaccines (now Pfizer), Sanofi-Pasteur and Merck. ML has accepted consulting fees and advisory meeting honoraria from Novartis and Pfizer, for work performed on matters not related to *Streptococcus pneumoniae*. LHM has received honoraria for DSMB and/or advisory boards for Merck, Pfizer, and Novartis. KLO and LHM serve on a *Streptococcus pneumoniae* vaccine advisory board for Merck. All other authors no conflicts.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.11.004.

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