

Nontypeable Pneumococcal Isolates Among Navajo and White Mountain Apache Communities: Are These Really a Cause of Invasive Disease?

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Background. Pneumococci could evade pneumococcal conjugate vaccines (PCV) by modifying, mutating, or deleting vaccine-serotype capsule genes or by downregulating capsule production. We sought to assess whether pneumococci that are nontypeable (NT) by the Quellung reaction truly lack capsule genes or are failing to produce capsule in vitro.

Methods. We applied multilocus sequence typing and a microarray for detection of pneumococcal polysaccharide capsule biosynthesis genes to NT carriage (children aged <5 years; years 1997–2000, 2006–2008) and NT invasive disease (IPD) (all ages; years 1994–2007) isolates from Native American communities.

Results. Twenty-seven of 28 (96.4%) NT IPD isolates had sequence types (STs) typically found among typeable IPD isolates and contained whole or fragments of capsule genes that matched known serotypes; 1 NT-IPD isolate had a profile resembling NT carriage isolates. Forty-nine of 76 (64.5%) NT carriage isolates had STs that typically lack capsule genes and were similar to NT carriage isolates found globally.

Conclusions. This is the first documentation of IPD from an NT strain confirmed to lack all known capsule genes. Most NT IPD isolates have or had the capacity to produce capsule, whereas a majority of NT carriage isolates lack this capacity. We found no evidence of pneumococcal adaptation to PCV7 via downregulation or deletion of vaccine-serotype capsule genes.

Streptococcus pneumoniae (pneumococcus) is the leading vaccine-preventable cause of morbidity and mortality among children aged <5 years [1]. Pneumococcal disease prevention through pneumococcal conjugate vaccine (PCV) use among infants and children is a major area of public health focus globally. Pneumococci are categorized into serogroups and serotypes

based on the immunological similarities of their polysaccharide capsule. The Quellung reaction, a standard method to serotype pneumococcal isolates, uses antisera against serotype-specific polysaccharide capsule [2]. However, there are pneumococcal isolates that cannot be typed by this method due to lack of capsule production; these are termed nontypeable (NT) pneumococci.

There are some ecological advantages for pneumococci to not produce capsule. A process called phase variation has been described in the pneumococcus, in which transparent variants show greater adherence to epithelial cell walls than opaque variants [3, 4]. Transparent variants decrease their expression of capsule and increase their expression of surface attachment proteins, assisting in attachment to epithelia and supporting colonization of the nasopharynx. In contrast, opaque variants are more associated with invasive

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disease and express higher levels of capsule, which conceal immunogenic surface proteins from the host. Lack of capsule production can occur by either downregulating gene expression of existing functional capsule polysaccharide biosynthesis (*cps*) genes or by the acquisition of genetic lesions, such as single nucleotide polymorphisms (SNPs), or deletion of *cps* genes, which renders capsule polysaccharide biosynthesis non-functional. Most genes required for capsule polysaccharide biosynthesis are located in a genomic locus flanked by *dexB* and *aliA* genes and include shared and serotype-specific gene sequences [5–7].

Most published research on NT pneumococci has been from conjunctivitis outbreaks from which NT strains are routinely isolated [8–13]. A small number of NT pneumococcal carriage studies have also been conducted [14, 15]. Two distinct groups of NT carriage isolates have been described using the multilocus sequence typing (MLST) method [8, 14–16]. One group contains a full complement of *cps* genes for a particular serotype, and the rest of the genome is closely related to known serotypeable lineages. The other group is not closely related to serotypeable pneumococci and contains no functional capsule genes. The NT pneumococci of the second group are common in conjunctivitis outbreaks and appear regularly among carriage isolates. It is generally believed that all pneumococci causing invasive pneumococcal disease (IPD) contain functional capsule genes and that IPD from strains that do not have the ability to produce capsule does not occur; however, there are extremely limited genetic data on NT IPD isolates. A small MLST analysis of 7 NT IPD and 20 NT carriage isolates identified 2 distinct groups, 1 related to “encapsulated” serotypeable strains, and the other related to NT strains typically found in conjunctivitis outbreaks [16].

Pneumococcal conjugate vaccines, the current approach to disease prevention in infants and children, are capsule-based products. Establishing whether NT pneumococci, especially those found in IPD, lack capsule gene loci or are simply failing to produce capsule in vitro is essential. Four percent of IPD isolates obtained from infants across the United States were NT by Quellung in 2004 [17]. Downregulation of meningococcal serogroup C capsule production has been described in serogroup C meningococci isolated from populations who had received conjugate meningococcal serogroup C vaccine in the United Kingdom between 1999 and 2001 [18]. Capsule expression in other meningococcal serogroups remained constant during the time period. The adaptive abilities of the pneumococcus through recombination and horizontal gene transfer with other pneumococci or related species in the nasopharynx may give it the capacity to adapt to vaccine pressure by switching or deleting serotype-specific capsule gene loci.

Analysis of NT pneumococci is particularly important in Navajo and White Mountain Apache communities in which there are high rates of pneumococcal carriage and disease and

high PCV7 coverage [19, 20]. Three percent (95% confidence interval [CI], 1.7%–4.1%) of IPD isolates obtained from Navajo children and adults between 2001 and 2006 [19] and 5% (95% CI, 4.2%–5.7%) of pneumococcal carriage isolates from Navajo and White Mountain Apache children collected between 2006 and 2008 were NT [20]. In contrast, only 1.88% (95% CI, .1%–.5%) of carriage isolates from children aged <7 years were NT in a study conducted in Massachusetts [21].

This study characterized pneumococci that were NT by the Quellung reaction from IPD and carriage samples collected from Navajo and White Mountain Apache communities. We aimed to describe their relatedness using MLST, to determine the presence of *cps* genes by microarray, and to assess any significant changes in these genes that may have occurred since introduction of PCV7. We expected the IPD isolates to contain genes for capsular biosynthesis and otherwise closely resemble serotypeable isolates by MLST. Conversely, we expected the carriage isolates to lack capsule genes and be comprised of sequence types that are typically NT.

METHODS

Pneumococcal carriage isolates were collected from Navajo and White Mountain Apache children aged <5 years who were enrolled in a PCV7 efficacy trial (1997–2000) [22] or enrolled in a study of nasopharyngeal (NP) colonization within families after routine use of PCV7 (2006–2008) [20]. The IPD isolates were obtained from population-based active surveillance of clinical microbiology laboratories serving these same communities [19]. Methods to obtain the NP and IPD specimens are described elsewhere [19, 23]. Morphology and alpha-hemolysis were the initial criteria used to select colonies from inoculated trypticase soy agar plates with 5% sheep blood agar and gentamicin (Becton Dickinson). Pneumococcus was confirmed by optochin sensitivity and bile solubility assays. If pneumococcus was isolated from the sample, serotype was assessed using the standard Quellung reaction.

We defined isolates as being NT if the isolate could not be typed by the Quellung reaction on 2 attempts for IPD isolates and a single attempt for NP isolates. From the latter longitudinal NP study, this analysis only included 1 NT carriage isolate per household to avoid over-representing NT strains circulating within households. All NT IPD isolates identified from 1994 through 2007 from Navajo and White Mountain Apaches of all ages were included in the analysis [19].

MLST

Pneumococci were prepared for MLST and sequence types determined by MLST using described methods [24, 25]. Polymerase chain reaction products were sequenced using a Prism 3730 × I Genetic Analyzer (Applied Biosystems). The raw sequences were analyzed using Molecular Evolutionary Genetics

Analysis 4 software (<http://www.megasoftware.net>). Alleles and sequence types were assigned using the online MLST database (<http://spneumoniae.mlst.net>). The eBURST algorithm (version 3, <http://eburst.mlst.net>) was used to group sequence types into clonal complexes (CCs) composed of closely related sequence types [26]. To determine whether the sequence type was previously associated with a particular serotype(s), we searched the MLST database [27].

Molecular Serotyping

All IPD isolates and a random subset of carriage isolates were characterized by a microarray assay to determine the presence of known *cps* genes [28]. 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 × 15 K platform (Agilent Technologies) [28]. The microarray included reporters to represent all *cps* genes involved in capsule polysaccharide biosynthesis of the 91 serotypes known to date [5, 29, 30]. The serotype was determined by the combination of *cps* genes found to be present in the isolate by microarray analysis. Pneumococcal samples for microarray were prepared using Qiagen DNeasy Mini Spin Column kits per manufacturer's instructions.

Analysis of PCV7 Adaptation

To assess whether downregulation of PCV7 serotype capsule genes has occurred more frequently since introduction of PCV7, we compared the proportion of PCV7 serotypes identified by microarray among the NT pneumococci before and after PCV7 introduction using a Fisher's exact test. If this mode of adaptation to PCV7 was occurring, we would expect to see an increased proportion of PCV7 serotypes detected by microarray among the NT pneumococci since introduction of the vaccine. We also compared the proportion of sequence types associated with PCV7 serotypes [27] before and after PCV7 introduction, again using a Fisher's exact test. An increase in sequence types associated with PCV7 serotypes among the NT strains after PCV7 introduction would suggest adaptation to vaccine pressure via reduction in capsule production. All statistical analyses were performed in STATA 9.1.

RESULTS

Sequence Type Analysis and Clustering Unique to Carriage Isolates

A total of 104 NT pneumococci were analyzed by MLST, 28 from IPD episodes and 76 from NP carriage events. The sequence types (STs) of the isolates, year and source of isolation, and serotypes commonly associated with that particular ST according to the MLST online database are found in the Appendix. Among the 76 carriage isolates, 25 STs were identified. Forty-nine of the 76 (64.5%) NT carriage isolates were

sequence types ST344 (n = 17), ST448 (n = 13), ST1054 (n = 9), ST1186 (n = 2), and ST2011 (n = 8), which are almost always identified as NT strains [26]. Thirteen novel STs were identified among the carriage isolates. Within these novel STs, strains 345, 417, 1418–08, and 2141–08 also each had 1 novel allele sequence that had not been previously identified in the MLST database.

Among the 28 NT IPD isolates, 16 STs were identified. Sequence types 191 (n = 4) and 227 (n = 5) were the 2 most common STs found among these NT IPD isolates, and they were not found among the NT carriage isolates. Sequence types 191 and 227 are commonly found among typeable IPD isolates from this population and others [27, 31]. Only 1 IPD strain (strain 1618) was identified as ST1054, which is an ST typically associated with NT carriage strains [15]. A sequence type for IPD strain 1617 could not be determined. The primers failed to work for the *aroE* and *spi* alleles after multiple attempts, and the other alleles were novel, suggesting this isolate was actually not a pneumococcus, but a closely related species.

The eBURST diagram in Figure 1 illustrates the relationship and differences between STs found in IPD and carriage. Only ST1840 and ST1054 were found in both IPD and carriage. There were 2 ST1840 IPD isolates and 3 ST1840 carriage isolates. This ST is generally associated with serotype 16F and NT strains. Sequence type 1054 is generally associated with NT carriage isolates, and 9 of 10 of our ST1054 isolates were indeed from carriage. However, 1 ST1054 isolate was found in IPD, and the microarray analysis revealed this isolate did not contain a *cps* locus, as typical to ST1054 NT carriage isolates. Sequence types found among IPD isolates did not form any CCs with other STs found in this NT dataset, whereas there were 6 CCs identified among our carriage isolates. Of the 6 founders of these CCs, ST344, ST448, ST1186, and ST2011 are types that are generally only associated with NT strains and not found among strains with capsule [14, 15].

Presence of *cps* Genes in NT IPD Isolates

The microarray analysis of the 28 NT IPD isolates revealed that 20 isolates had *cps* gene content fully matching the capsule gene loci of known serotypes, whereas 8 had capsule gene content that only partially matched capsule gene loci of known serotypes (Table 1). The serotypes predicted by microarray corresponded to the serotypes associated with the MLST analysis in all but 1 case (strain 1634). This strain was ST218, which is generally associated with serotype 12F; however, serotype 7F capsule genes were found on the microarray. Strain 1626 had an ST199, typically associated with serotype 19A; however, the microarray analysis identified capsule genes for both serotype 19A and serotype 37. The intensity measured for serotype 19A was much stronger, suggesting a serotype 19A sample was mixed or contaminated with serotype 37 during the isolation or culturing process. We failed to

Table 1. Array Serotype, Source, Year of Isolation, Sequence Type (ST), and Associated Serotypes of Nontypeable (NT) Pneumococcal Isolates^a

Strain ID	Source	Year	Array Serotype	ST	Associated Serotypes ^b
1620	IPD	1990	9N	66	9N, 19F, 14
1614	IPD	1994	18C	113	18C
1613	IPD	1994	7F	191	7F
1610	IPD	1994	4	247	4
1611	IPD	1994	4	247	4
1616	IPD	1995	14	124	14
1615	IPD	1995	7F	191	7F
1612	IPD	1995	5	3035	5
1617	IPD	1995	NT (non-pneumoniae): 16A-like but <i>wzg</i> , <i>wzh</i> , <i>wzd</i> , <i>wze</i> , <i>wchA</i> , <i>rmlD</i> absent or divergent	Failed	NA
1618	IPD	1998	NT: only <i>glf</i> present	1054	NT
1619	IPD	1999	14	124	14
1626	IPD	2002	19A (92%) + 37 (8%)	199	19A and others
1636	IPD	2002	NT: only <i>rmlB</i> , <i>rmlD</i> , <i>rmlA</i> , <i>rmlC</i> present	227	1
1635	IPD	2003	7F	191	7F
1625	IPD	2003	37	447	37, 11
1622	IPD	2003	NT: 22A-like but <i>wcwC</i> and <i>wcwA</i> absent or divergent	1294	22F
1627	IPD	2003	33A/F	1367	33F
1623	IPD	2003	16F	1840	16F
1628	IPD	2004	1	227	1
1621	IPD	2004	NT: only <i>rmlB</i> , <i>rmlD</i> , <i>rmlA</i> , <i>rmlC</i> present	227	1
1629	IPD	2004	NT: only <i>rmlB</i> , <i>rmlD</i> , <i>rmlA</i> , <i>rmlC</i> present	227	1
1633	IPD	2004	8	1480	8
1631	IPD	2005	8	1480	8
1632	IPD	2005	NT: 16F-like but <i>wzg</i> gene absent or divergent	1840	16F
1624	IPD	2006	7F	191	7F
1634	IPD	2006	7F	218	12F
1630	IPD	2006	NT: only <i>rmlB</i> , <i>rmlD</i> , <i>rmlA</i> , <i>rmlC</i> present	227	1
1637	IPD	2007	33A/F	100	33F
2566-06	NP	2006	NT: only <i>glf</i> present	448	NT
2537-06	NP	2006	35F	487	35F
2489-06	NP	2006	NT: no <i>cps</i> genes detected	2011	NT
2563-06	NP	2006	35A	4505	None
0900-07	NP	2007	NT: only <i>glf</i> present	344	NT
2009-07	NP	2007	34	547	34
1944-07	NP	2007	6C	3101	6A
1878-08	NP	2008	NT: only <i>glf</i> present	1054	NT
1927-08	NP	2008	35A	3026	35A

Abbreviations: IPD, invasive pneumococcal disease; NA, not available; NP, nasopharyngeal.

^a Sorted by source, year of isolation, and ST.

^b Per Multilocus Sequence Typing online database.

carriage isolates revealed that the typically NT pneumococci (ie, ST344, ST448, ST1054, and ST2011) either had only *glf* present or no known capsule genes (Table 1). Therefore none would be able to produce capsule. The STs that were associated with specific serotypes had complete *cps* genes present for the expected serotype, suggesting that they were either not expressed in vitro or were not detected by Quellung.

No Evidence of Pneumococcal Adaptation to PCV7 by Downregulation or Deletion of Capsule Genes

The MLST and microarray analysis of our NT isolates do not show evidence that pneumococci in this population are adapting to PCV7 pressure by capsule loci deletion or downregulation, although the analysis was limited in power. Among NT IPD isolates from prior to routine PCV7 introduction in 2000,

45% had PCV7 serotype capsule genes by microarray and 55% had PCV7-associated STs, whereas none of the NT IPD isolates after PCV7 introduction (2001–2007) had PCV7 serotype capsule genes or STs. Furthermore, no NT carriage isolate had a PCV7 ST or microarray serotype regardless of the isolation date. This analysis provides additional evidence that the vast majority of NT IPD isolates resemble serotypeable IPD isolates in the makeup of their genetic material. We infer they were likely expressing capsule *in vivo* and were therefore eliminated from invasive disease and carriage after PCV introduction.

DISCUSSION

This study has described 4 groups of NT isolates in a defined population: (1) isolates that lack known *cps* gene sequences (or only had the *glf* gene present); (2) isolates that have partial *cps* loci; (3) isolates with complete *cps* loci when compared with known serotypes; and (4) isolates that are not likely to be pneumococci (strain 1617). We consider the first 2 types to be truly NT pneumococci because they are deficient in the ability to produce capsule, whereas those of the third type likely retain the capability of capsule production but are not expressing capsule *in vitro*. Identifying non-pneumococci via microarray is not uncommon in pneumococcal carriage studies (J. Hinds, personal communication) and is important because these related species may also contribute *cps* or antimicrobial resistance genes to the gene pool available for genetic exchange in the nasopharynx. However, isolate purification or contamination must be ruled out before exploring the possibility that this non-pneumococcal isolate had the potential to cause invasive disease.

We conclude that this collection of NT IPD isolates fundamentally differs from the majority of NT carriage pneumococcal isolates in this population and others. Only 1 NT IPD isolate, strain 1618 (ST1054), had an MLST and array profile that suggests true NT IPD and resembles NT carriage isolates. All other STs identified among the NT IPD isolates have routinely been found among serotypeable IPD isolates in this population [31]. Most of the NT IPD isolates studied also have complete *cps* genes present for known serotypes, suggesting that capsule production was simply downregulated *in vitro*, thereby resulting in a negative Quellung reaction. A few NT IPD isolates were either missing or had divergent *cps* genes as identified by the microarray and appear to be remnants of known serotypes. No pneumococci isolated after routine PCV7 introduction had PCV7 serotype genes by microarray, mirroring the reduction observed in PCV7 serotype IPD rates by Quellung [19].

In contrast, the NT carriage strains we tested were comprised of either isolates with complete *cps* gene loci, which were likely downregulated *in vitro*, or isolates with either no known capsule genes present or only *glf*, both typical of truly

NT pneumococci isolates. Both carriage groups have STs typically found among the other serotypeable carriage isolates from this population [31] and include STs that have been described in other NT carriage studies [14–16]. We did not find any NT carriage isolate with only a partial *cps* gene sequence; however, the small number of carriage isolates we analyzed by microarray limits this information.

The few isolates with partial known *cps* loci were unique to the IPD isolates and likely lacked specific genes required to produce serotype-specific antigen targeted in the Quellung reaction. The missing serotype 1 genes in strains 1621, 1629, 1630, and 1636 (all ST227) are usually located in a contiguous region in serotype 1 that is flanked by 2 insertion sequence elements (IS1167), suggesting that the deletion seen in all 4 strains may have been transposase or recombination mediated. The isolates were collected between 2002 and 2006 from 3 adults and 1 child living in 3 geographically distinct areas. The presence of insertion sequence elements and their diverse geographies and time periods suggest the isolates may have emerged independently. Further exploration would be necessary to determine whether these deletions may have occurred *in vivo* or *in vitro*.

Some NT isolates may simply be identified as such because of imperfect pneumococcal typing methods. Clarifying the likelihood that isolates are truly NT will improve the quality of pneumococcal typing efforts that are critical to pneumococcal disease surveillance and vaccine development. There may also be explanations for why isolates with complete *cps* loci were NT, technical errors aside. Single nucleotide polymorphisms in the gene sequence have been shown to truncate *cps* gene products, which in turn prevent capsule production [32]. These isolates are NT by the Quellung reaction, but the gene sequences are likely similar enough to be detected on the microarray. Small deletion(s) or SNPs could be identified by sequencing and compared with known complete *cps* gene sequences for a specific serotype; their effect on *cps* gene expression could be assessed by transcriptional analysis by microarray; and their effect on *cps* production could be measured by an enzyme-linked immunosorbent assay [4].

Impact and Future of NT Pneumococci

This study affirms the widespread dissemination of NT pneumococcal STs that lack capsule gene loci in carriage and the near absence of these particular STs in IPD. It does appear that true NT IPD can occur, albeit very rarely. Capsule is clearly not required for survival in and transmission between the nasopharynx and other mucosal membranes, such as the conjunctiva. Our study found no evidence of pneumococcal adaptation to PCV7 by downregulation or deletion of PCV7 serotype capsule genes after 8 years of routine vaccine use, although our analysis was limited in power. We confirmed that there were no STs or array serotypes associated with PCV7

serotypes in the vaccine era among the NT IPD or carriage isolates, mirroring what has been observed among the isolates identified as vaccine serotype by conventional means [20, 31].

Nontypeable IPD isolates identified in future studies should be further analyzed beyond serotyping to determine what capsule genes are present and which appear necessary for invasive disease. Although rare in this study, NT IPD isolates with incomplete capsule gene loci would also require characterization of capsule products and assessment of whether deletions occurred in vivo or in vitro postisolation. Distinguishing the role of specific capsule genes could help direct additional disease prevention efforts and identify adaptive mechanisms to current ones.

Notes

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All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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