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## The Nonserotypeable Pneumococcus: Phenotypic Dynamics in the Era of Anticapsular Vaccines<sup>∇</sup>

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**Nonserotypeable pneumococci (NSP) are commonly carried by Australian Indigenous children in remote communities. The purpose of this study was to characterize carriage isolates of NSP from Indigenous children vaccinated with the seven-valent pneumococcal conjugate vaccine (PCV7) and to use these data to guide decisions on reporting of NSP. A total of 182 NSP were characterized by BOX typing, antibiogram analysis, and multilocus sequence typing (MLST) of common BOX types. NSP positive for the *wzg* capsule gene were analyzed by a multiplex PCR-based reverse line blot hybridization assay (mPCR/RLB-H) targeting capsule genes to determine the serotype. Among 182 NSP, 49 BOX types were identified. MLST of 10 representative isolates found 7 STs, including ST448 (which accounted for 11% of NSP). Non-penicillin susceptibility was evident in 51% of the isolates. Pneumococcal *wzg* sequences were detected in only 23 (13%) NSP, including 10 that contained an ~1.2-kb insert in the region. mPCR/RLB-H identified serotype 14 *wzy* sequences in all 10 NSP, and 1 also contained a serotype 3-specific *wze* sequence. Among the remaining 13 *wzg*-positive NSP, few belonged to the serotypes represented in PCV7. It appears that most NSP identified in Australian Indigenous children are from a true nonencapsulated lineage. Few NSP represented serotypes in PCV7 that suppress capsular expression. High rates of carriage and penicillin resistance and the occasional presence of capsule genes suggest a role for NSP in the maintenance and survival of capsulated pneumococci. To avoid the inflation of pneumococcal carriage and antibiotic resistance rates, in clinical trials, we recommend separate reporting of rates of capsular strains and NSP and the exclusion of data for NSP from primary analyses.**

Australian Indigenous children in remote communities of the Northern Territory experience dense, persistent nasopharyngeal colonization with *Streptococcus pneumoniae* (pneumococcus) from early infancy (13, 25). Cross-sectional studies report pneumococcal carriage rates of over 80% for these children (15). Pneumococcal serotype diversity contributed to swift serotype replacement following introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) in 2001. The vaccine had no significant impact on the incidence of overall pneumococcal carriage or otitis media (14).

To date, 91 pneumococcal serotypes have been described (21). In addition, a population of nonserotypeable pneumococcal (NSP) isolates does not react with the capsular polysaccharide typing sera. Molecular typing has identified three categories of NSP: (i) pneumococci that lack capsule genes, (ii) pneumococci that possess capsule genes but that are phenotypically nonencapsulated, and (iii) atypical pneumococci that are phenotypically NSP but that are genetically

divergent from pneumococci (8,18). Pneumococci that lack capsule genes make up a highly diverse population that includes strains that have spread globally (8). These strains have been associated with a variety of mucosal (and, occasionally, invasive) diseases (for a review, see reference 24). Phenotypically nonencapsulated pneumococci that possess capsule genes may represent pneumococci that have lost the ability to express the capsule or strains that have temporarily ceased or downregulated capsule expression. Understanding the distinction between these populations is particularly important in the context of widespread immunization with anticapsular vaccines.

NSP frequently colonize Indigenous Australian children. In our studies with young Indigenous children, NSP were detected in up to 18% of nasopharyngeal swab specimens. In a recent cross-sectional carriage study, NSP were the 3rd most common pneumococcal serotype (unpublished data from reference 16). It is also likely that we underestimate NSP carriage rates because of their morphological differences from their capsular counterparts; NSP tend to be smaller and dryer than capsular pneumococci, and the dimple is less conspicuous. The purpose of this study was to characterize NSP carriage isolates collected from Indigenous children after the introduction of PCV7. Our aim was to understand the potential importance of this population, particularly with regard to the presence of the capsule genes and antibiotic resistance. Importantly, we require evidence to guide reporting of NSP carriage and resis-

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TABLE 1. Carriage study details

Study	Description (reference) <sup>a</sup>	Yr(s)	No. of subjects enrolled	No. of swabs (nasal or nasopharyngeal)	Age at enrollment (mo)	
					Median	Range
1	Longitudinal carriage study of infants receiving PCV7 and 23PPV <sup>a</sup> in three communities (17)	2001–2004	97	914 (two to four weekly to 1 yr, 18 mo, and 24 mo)	1.13	0.23–7.96
2	RCT of azithromycin vs amoxicillin for AOM in 16 communities (in press)	2003–2005	320	661 (days 0, 6–11, and 12–21 if AOM with perforation was present)	13	5.9–71.8
3	Cross-sectional carriage surveys in four Indigenous communities <sup>b</sup> (16)	2002	192	212	85.7	24–188
4	Cross-sectional carriage surveillance of children ages 0 to 6 yr in 29 (2003) and 17 (2005) communities (15)	2003 2005	902 818	902 818	21.3 33.7	0.76–75 0.36–72

<sup>a</sup> 23PPV, 23-valent pneumococcal polysaccharide vaccine; RCT, randomized controlled trial; AOM, acute otitis media.

<sup>b</sup> Children <16 years of age were included.

tance in pneumococcal surveillance studies and as microbiological outcomes in clinical trials for otitis media.

#### MATERIALS AND METHODS

**Ethics.** Ethical approval for this study was granted by the Human Research Ethics Committee of the Northern Territory Department of Health and Families and the Menzies School of Health Research. Isolates were selected only if the parents or guardians consented at enrollment to the samples being used for future research.

**Definition of NSP.** NSP were grown on horse blood agar plates (Oxoid, Australia) at 37°C in 5% CO<sub>2</sub> and identified as optochin sensitive, alpha-hemolytic colonies with a negative reaction to Quellung omniserum (1) and a positive autolysin (*lytA*) PCR result (19).

**Samples.** A total of 182 nasopharyngeal NSP isolates were randomly selected (Research Randomizer; <http://www.randomizer.org>) from four studies of otitis media (Table 1) conducted in the Northern Territory after the introduction of PCV7. The isolates were stored at –80°C in skim milk-tryptone-glucose-glycerol broth (20). When the specimens originated from longitudinal studies, sample selection was limited to the first NSP isolate recovered from each child.

**Antibiotic susceptibility testing.** Screening for penicillin and macrolide susceptibility was determined by a calibrated dichotomous susceptibility (CDS) disk diffusion assay (oxacillin and erythromycin, respectively) (3). Etests (AB Biodisk, Solna, Sweden) were used to determine the penicillin and erythromycin MICs of all nonsusceptible isolates (CDS radius < 6 mm). PCR for the detection of macrolide resistance genes (*mefA/E* and *ermB*) was performed as described previously (18, 26).

**DNA extraction and quantification.** NSP DNA for BOX typing and the detection of macrolide resistance genes was extracted by using the Instagene matrix (Bio-Rad), according to the manufacturer's instructions. NSP DNA for multilocus sequence typing (MLST) was extracted with a QIAamp DNA minikit (Qiagen), according to the manufacturer's instructions. The DNA concentration was determined with a Picogreen double-stranded DNA quantitation assay kit (Molecular Probes). The extraction of DNA for multiplex PCR/reverse line blot hybridization (mPCR/RLB-H) was done as described previously (12).

**Capsule gene PCR.** The capsule gene was detected by the method of Hanage et al. (8), with the exception that primers *wzg*-up (5'-ATCCTTGTCAGCTCTGTGTC) and *wzg*-down (5'-TCACTTGCAACTACATGAAC) amplified a 1,797-bp product.

**Molecular typing.** BOX typing was done as described previously with 1 ng of DNA in each 50- $\mu$ l reaction mixture (27). The BOX types were defined according to the banding patterns between 750 bp and 3,000 bp. MLST was undertaken essentially as described previously (6), except for modification of the *spi* forward primer (primer *spiF3*, 5'-CAGATTTTGCAAGCCTATGG), the *recP* reverse primer (primer *recPR2*, 5'-GCCGTGTACAGCATTAGTTC), and the *ddl* forward primer (primer *ddlF2*, 5'-GATGGCTCTGTTC AAGGATT). Molecular serotyping of *wzg*-positive isolates of NSP was by done mPCR/RLB-H, as described previously (11, 28), for 90 pneumococcal serotypes. This method targets serotype-specific regions of *wzy* for all serotypes with the exception of serotype 3. In the case of serotype 3, *wze* is targeted, as *wzy* is not present in serotype 3 strains.

#### RESULTS

**NSP colonization of Indigenous children.** Presumptive NSP in this study were identified on the basis of colonial morphology, optochin sensitivity in 5% CO<sub>2</sub> for distinction from *S. pseudopneumoniae* (2), and a negative Quellung reaction. In longitudinal studies 1 and 2 (Table 1), presumptive NSP were reported in 18% and 8% of the nasopharyngeal swab specimens, respectively. Cross-sectional studies 3 and 4 reported presumptive NSP carriage prevalence rates of 8% and 5%, respectively. These numbers are likely to underestimate the true carriage rate because NSP were not systematically sought. Variations in the rates reported between studies may also be due to subjectivity in the selection of colonies.

**Characterization of NSP by BOX typing and antibiogram analysis.** Among the 182 randomly selected isolates of NSP (which were confirmed to be *lytA* positive), 49 BOX types were identified. The three most common BOX types (types 1, 14, and 3) represented 44% of the isolates. BOX types 1 and 14 were closely related, distinguished by a single band size difference of less than 50 bp.

Intermediate resistance to penicillin (MICs, 0.12 to 1  $\mu$ g/ml; breakpoint for oral therapy of nonmeningococcal strains) was evident in 49% (90/182) of the NSP isolates, penicillin resistance (MICs,  $\geq 2$   $\mu$ g/ml) was detected in 2% (4/182) of the isolates, and erythromycin resistance (MICs,  $\geq 4$   $\mu$ g/ml) was detected in 10% (19/182) of the isolates. Erythromycin resistance was mediated by the *mefE* macrolide resistance gene in all except one of the isolates; the latter isolate was negative for the *mefA/E* and *ermB* genes. Reduced susceptibility to these commonly used antimicrobials was detected in isolates from 32 BOX types.

**Characterization of NSP by MLST.** Representative isolates of the three most common BOX types, including all antibiograms, were analyzed by MLST (Table 2). BOX type 1 and 14 isolates were represented by six sequence types (STs), all of which were new to the MLST database. Two STs were common to these BOX types. ST4485 was a double-locus variant of a single ST corresponding to a serotype 4 isolate. ST4487 and ST4488 were both double-locus variants of two STs corresponding to serotype 14 and 19A isolates. The BOX type 3 isolate was ST448, an NSP clone reported internationally.

TABLE 2. MLST investigation of the three most common NSP BOX types

BOX type	No. (%) of isolates	MLST type (no. of isolates)
1	34 (19)	4485 (1)
		4486 (1)
		4487 (1)
		4488 (1)
14	25 (14)	4484 (1)
		4486 (1)
		4488 (2)
		4565 (1)
		448 (1)
3	20 (11)	

ST448 and STs 4486, 4484, and 4565 were not closely related (less than five identical MLST loci) to STs corresponding to capsular pneumococci that are currently in the MLST database. All isolates were confirmed to be pneumococci by using the “is it a pneumococcus?” analysis ([www.mlst.net](http://www.mlst.net)).

**Presence of *wzg* gene.** As described previously (8), we used PCR for the conserved *wzg* (*cpsA*) gene to test for the presence of a *cps* locus (Tables 3 and 4). *wzg* was detected in 23 of 182 (13%) NSP isolates and 13 BOX types. However, 10 isolates provided a product of approximately 3 kb rather than the expected 1.8-kb product. Partial sequencing of the product demonstrated a sequence homologous to that of the pneumococcal *wzg* gene and an unidentified insert which was not related to streptococcal *ali* genes, as reported previously (9). The 10 isolates represented four different BOX types, suggesting that this insertion was not a recent event.

mPCR/RLB-H analysis of the isolates with the expected 1.8-kb *wzg* gene determined that five isolates corresponded to PCV7 serotypes 9V (or 9A) and 14, whereas eight corresponded to non-PCV7 serotypes 7A/F, 16F, 11A/D, 22A/F, and 13 (Table 3). Cocolonization with a capsular strain of the corresponding serotype was evident for 8 of 13 NSP with the 1.8-kb *wzg* product (Table 3). Subsequent testing of all strains with specific type antisera found that none reacted.

mPCR/RLB-H analysis determined that the 10 isolates with the 3-kb *wzg* PCR product corresponded to serotype 14. Two BOX type 14 isolates positive for the insertion were ST4488 (double-locus variants of STs corresponding to serotype 14 and 19A isolates).

One isolate consistently provided a serotype 14 result (based on the result for *wzy*) and a serotype 3 result (based on the result for *wze*), suggesting mosaicism of the strain and that both serotype 3 and 14 capsular genes may exist in this strain.

Reduced susceptibility to antimicrobials was common in all groups (Table 4).

## DISCUSSION

Australian Indigenous children living in remote communities experience capsular pneumococcal carriage rates of approximately 80% and NSP carriage rates of 5 to 20%. These NSP carriage rates are likely to be underestimates because NSP are morphologically distinct from capsular pneumococci and NSP were not systematically sought in the studies that determined those carriage rates. NSP detection rates in a pop-

TABLE 3. Investigation of serotypes associated with *wzg*-positive NSP<sup>a</sup>

BOX type	MLST type	NSP serotype	Simultaneously carried serotypes
1.8-kb <i>wzg</i> product			
5		11A/11D <sup>b</sup>	11A
6		7A/7F <sup>b</sup>	7F
8		16F	16F
8		16F	16F
14		9A/9V <sup>b</sup>	15B
14		9A/9V <sup>b</sup>	None
14		<b>14</b>	19A
17		22A/22F <sup>b</sup>	22F
25		<b>14</b>	19F
30		<b>14</b>	<b>6B</b>
32		13	13
52		16F	16F
57		16F	16F
3-kb <i>wzg</i> product			
1		<b>14</b>	None
1		<b>14</b>	19F
1		<b>14</b>	None
13		<b>14</b>	21
14		<b>14</b>	19A
14		<b>14</b>	None
14		<b>14</b>	19A
14	4488 <sup>c</sup>	<b>14</b>	16F
14	4488 <sup>c</sup>	<b>14</b> and 3 <sup>d</sup>	None
15		<b>14</b>	15C

<sup>a</sup> The serotype of each *wzg*-positive NSP was determined by mPCR/RLB-H. Capsular pneumococcal serotypes simultaneously carried in each swab are shown. PCV7 serotypes are indicated in boldface.

<sup>b</sup> Pneumococcal serogroups indistinguishable by mPCR/RLB-H.

<sup>c</sup> Double-locus variant of STs corresponding to serotypes 14 and 19A.

<sup>d</sup> This isolate reacted with serotypes 14 and 3 by mPCR/RLB-H.

ulation appear to be dependent on the study protocol and subjectivity in colony selection.

Cocolonization with capsular and noncapsular pneumococci occurred frequently, providing an opportunity for an interaction between these populations. Previous studies of NSP report a highly diverse population with a pervasive nature, an ability to cause disease, and a likely important role in pneumococcal ecology (8, 23, 24). Although nonencapsulated forms are less likely to cause disease, their ability for gene transfer, including the transfer of antibiotic resistance and capsular/virulence genes, is predictably high due to their capacity for an increased rate of transformation (22).

**The majority of NSP are likely to be of the true nonencapsulated lineage.** Two distinct populations were detected in this study: NSP with and without the *wzg* (*cpsA*) gene. The majority (87%) of NSP in this study were *wzg* negative by PCR and were thus likely to be of the true nonencapsulated lineage. However, given that 10 of 23 *wzg* sequences were atypical due to an insertion, we cannot rule out the possibility that other isolates contained modified *wzg* sequences not detectable by our methods.

Our grant did not provide funding for MLST of all NSP isolates; however, MLST of representatives of the three most common BOX types identified authentic pneumococci, including six new STs and ST448 (which accounted for 11% of the NSP in this study). ST448 is a particularly interesting clone, in that it is internationally disseminated and has been associated with invasive disease (9).

TABLE 4. Summary of typing and antimicrobial susceptibility data for NSP

Genotype	No. of isolates	No. of PCV7 serotypes <sup>a</sup>	No. of BOX types	No. (%) of isolates	
				Penicillin resistant <sup>b</sup>	Erythromycin resistant <sup>c</sup>
wzg positive	13	5	10	IR, 4 (31); R, 0	2 (15)
wzg and insert positive	10	10	4	IR, 2 (20); R, 0	3 (30)
wzg negative	159	Not done	44	IR, 84 (53); R, 4 (2.5)	14 (9)

<sup>a</sup> As determined by mPCR/RLB-H.

<sup>b</sup> Intermediate resistance (IR) is a penicillin MIC of 0.12 to 1 µg/ml; resistance (R) is a penicillin MIC of ≥2 µg/ml.

<sup>c</sup> Erythromycin MIC of ≥4 µg/ml.

Identification of the majority of NSP in this study was based on colonial morphology, optochin susceptibility, and a positive *lytA* PCR result. Therefore, it is likely that these strains were *S. pneumoniae* rather than *S. pseudopneumoniae* (which are generally optochin resistant in the presence of increased amounts of CO<sub>2</sub>) (2) or other closely related *Streptococcus* spp. However, as *lytA* is not ideal as a sole genetic criterion for pneumococcal identification, in due course MLST analysis may identify some strains as atypical pneumococci (divergent at the seven MLST loci by >5%) (7).

**NSP do not represent a population of PCV7 types suppressing capsule expression.** Capsular genes (*wzg*) were detected in a minority of the NSP isolates. Ten of these isolates had an insertion in *wzg* which may have interrupted capsule expression, while 13 contained capsule genes related to both PCV7 and non-PCV7 serotypes. Additionally, among 182 isolates, only 15 were potentially downregulated PCV7 types. These data do not support the hypothesis that PCV7 types are suppressing capsule expression to avoid vaccine-induced immune clearance.

**NSP may act as a parking lot for antibiotic resistance genes.** Transformation is a common mechanism for the development of resistance to penicillin and macrolides in pneumococci (4, 5). As NSP have transformability rates up to 1,075-fold higher than those of capsulated strains (22), NSP may act as a vector for the transmission of resistance genes between viridans group streptococci and capsulated pneumococci. Indeed, a serotype 19F clone became increasingly non-penicillin susceptible to by the uptake of *pbp2x* gene fragments shared with NSP (10). Despite being present in low numbers, nonencapsulated forms of PCV7 serotypes are also likely to harbor the antibiotic resistance genes of their parent capsular vaccine serotypes and thus have the potential to transfer antibiotic resistance to newly emerging nonvaccine serotypes. Our 2005 surveillance of pneumococcal carriage by Indigenous children found that 14% of the isolates were non-penicillin susceptible (MIC ≥ 0.12 µg/ml) and that 6% of the isolates were azithromycin resistant (MIC ≥ 2 µg/ml) (15). The penicillin resistance data are in stark contrast to the 55.5% rate of non-penicillin susceptibility that we found in *wzg*-negative NSP and 26% in potentially revertible *wzg*-positive NSP. Thus, there is potential for the selection of existing capsular strains exhibiting antibiotic resistance and for the uptake of resistance genes by susceptible capsular pneumococci during exposure to NSP.

**Reporting NSP in surveillance and clinical trials.** High carriage rates, antibiotic resistance, and the presence of capsule genes suggest a role for NSP in the maintenance and survival

of capsular pneumococci. Until this role is better understood, we support continued reporting of NSP carriage rates. Since MLST is necessary to explicitly confirm the identity of nonserotypeable pneumococcal isolates, it will be necessary to analyze data obtained by using less rigorous methods of identification with caution.

In populations with high NSP carriage rates, combining the data for NSP and capsular pneumococci would inflate the rates of pneumococcal carriage and antibiotic resistance. Therefore, we recommend separate reporting of rates of carriage of NSP and capsular pneumococci in surveillance studies. When the microbiological outcomes of clinical trials are reported, we recommend the inclusion of only the capsular pneumococci in primary analyses.

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