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Invasive and Noninvasive *Streptococcus pneumoniae* Capsule and Surface Protein Diversity following the Use of a Conjugate Vaccine

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The 13-valent pneumococcal conjugate vaccine (PCV13) was introduced in the United States in 2010 for the prevention of invasive pneumococcal disease (IPD) and otitis media. While many studies have reported its potential efficacy for IPD, not much is known about the epidemiology of noninvasive disease following its introduction. We characterized the capsular types and surface protein genes of noninvasive pediatric pneumococcal isolates collected between 2002 and 2010 ($n = 1,058$) at Children's of Alabama following the introduction of PCV7 and tested a subset of noninvasive and previously characterized IPD isolates for the presence of the *pspA*, *pspC*, and *rrgC* genes, which encode protection-eliciting proteins. PCV7 serotypes had dramatically decreased by 2010 ($P < 0.0001$), and only 50% of all noninvasive infections were caused by the PCV13 capsular serotypes. Serotype 19A accounted for 32% of the noninvasive isolates, followed by serotypes 35B (9%), 19F (7%), and 6C (6%). After 7 years of PCV7 usage, there were no changes in the frequencies of the *pspA* or *pspC* genes; 96% of the strains were positive for family 1 or 2 *pspA* genes, and 81% were also positive for *pspC*. Unexpectedly, more noninvasive than invasive strains were positive for *rrgC* ($P < 0.0001$), and the proportion of *rrgC*-positive strains in 2008 to 2010 was greater than that in 2002 to 2008 (IPD, $P < 0.02$; noninvasive, $P < 0.001$). Serotypes 19F, 19A, and 35B were more frequently *rrgC* positive ($P < 0.005$) than other serotypes. A vaccine containing antigens, such as PspA, PspC, and/or RrgC, can provide coverage against most non-PCV13-type pneumococci. Continued surveillance is critical for optimal future vaccine development.

Streptococcus pneumoniae is a major cause of morbidity and mortality worldwide due to pneumonia, bacteremia, and meningitis. Pneumococcal infections are estimated to cause 826,000 deaths globally in children <5 years of age (1) and result in billions of dollars in health care costs in the United States (2). Furthermore, it is estimated that 70 to 80% of severe pneumonia cases in Africa are caused by *S. pneumoniae* (3, 4) strains that are predominantly of capsular serotypes 1 and 5 (5). The introduction of the heptavalent pneumococcal conjugate vaccine (PCV7) has led to sufficient protection against colonization to almost completely eliminate invasive disease caused by the seven PCV capsular types (4, 6B, 9V, 14, 18C, 19F, and 23F), which caused the majority of invasive pneumococcal diseases (IPDs) prior to 2000. However, only a few years after the introduction of PCV7, serotype replacement (an increase in the incidence of IPD caused by non-PCV7 capsular types) was observed (6–10). In 2010, a new 13-valent vaccine was introduced in the United States to provide protection against the original PCV7 serotypes plus 6 additional capsular serotypes (1, 3, 5, 6A, 7F, and 19A) known to cause IPD. PCV13 has also been licensed for use against otitis media (11). Previous reports have shown that PCV13 protects against approximately 60 to 70% of all pneumococci globally postintroduction of the PCV7 (6, 12–14), and nonvaccine serotypes have been reported to cause serious diseases, such as meningitis (6, 8). The occurrence of serotype replacement only a few years following the introduction of PCV7 and the fact that there are >90 known capsular serotypes (15) raise concerns about the long-term usage of capsule-based vaccines.

One potential strategy to reduce serotype replacement is the inclusion of protein vaccine immunogens to provide protection that is not dependent on the antibody responses to capsular polysaccharides. Several candidate protein antigens exist, and previous studies have indicated that combinations of protein immunogens might provide a way to protect against a wider number of serotypes and diseases (16–19). The surface virulence proteins of pneumococci, in particular, are important nonpolysaccharide vaccine candidates. Pneumococcal surface protein A (PspA) is a protein on virtually all pneumococci (6, 20) and is important for the inhibition of the classical complement pathway, working specifically by competing with binding by C-reactive protein to block complement deposition (21). PspA elicits antibody-mediated protection against invasive infection through its alpha-helical regions. Moreover, the alpha-helical regions within, and frequently between, different PspA families are cross-reactive and cross-protective (22–26).

Another important surface protein is pneumococcal surface protein C (PspC; also known as CbpA), which has structural features similar to those of PspA and has been demonstrated to bind factor H, thus inhibiting the alternative pathway of complement deposition (27–30). PspC has also been shown to be important for adherence through its interaction with the polymeric Ig receptor (31, 32) and binding of secretory IgA (27). Mice immunized with PspC have been demonstrated to develop protective antibodies against pneumonia, sepsis, and colonization, and immunization with the PspC proline-rich region was found to be cross-protective against a pneumococcal strain lacking the *pspC* gene (33–37).

Pili were recently discovered in pneumococci (38–40). Pilus type 1, encoded by the *rlaA* islet, is an adhesin that binds to epithelial cells (41, 42). The major subunits, *rrgA*, *rrgB*, and *rrgC*, are all able to elicit active and passive protection (43), and *rrgC* is highly conserved

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TABLE 1 Primers used in this study

Primer name	Gene	Sequence (5′ to 3′)	Reference or source
LSM12	*pspA*	CCGGATCCAGCGTCGCTATCTTAGGGGCTGGTT	49
SKH63	*pspA* (family 1)	TTTCTGGCTCATYAACTGCTTTC	49
SKH52	*pspA* (family 2)	TGGGGGTGGAGTTTCTTCTTCATCT	49
ABW13	*pspC* fragment	CGACGAATAGCTGAAGAGG	35
SKH2	*pspC* fragment	CCACATACCGTTTTCTTGTTTCCAGCC	35
pspC_gates1	*pspC* full length	GAAAAAATATAGAAGGAAATAAAC	This study
pspC_gates2	*pspC* full length	CAGTATTAACTAGTTATATTAGG	This study
C5	*rrgC*	GCTCTGTGTTTTTCTCTTGTATGG	45
C3	*rrgC*	ATCAATCCGTGGTCGCTTGTTATTTTTA	45
plyF	*ply* fragment	ATTTCTGTAACAGCTACCAACGA	48
plyR	*ply* fragment	GAATTCCCTGTCTTTTCAAAGTC	48

among all serotypes of pneumococci with pilus type 1 (44, 45). Furthermore, for pneumococci that are piliated, immunity to pili has been shown to be protective against sepsis and colonization (43).

In this study, we present data on the capsular serotype distribution of pneumococci collected ($n = 1{,}058$) between 2002 and 2010 from noninvasive sites in pediatric patients. We also determined the gene frequencies of the pneumococcal protein-based vaccine candidates *pspA*, *pspC*, and *rrgC* (pilus type 1) in a subset of noninvasive isolates (this study) and previously characterized IPD isolates (6).

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MATERIALS AND METHODS

Data and patient selection. All viable pneumococci from sequential routine clinical specimens submitted to the Clinical Microbiology Laboratory at Children's of Alabama in Birmingham, AL, between July 2002 and June 2010 were collected prospectively from patients aged 0 to 18 years old. The site of isolation, clinical disease diagnosis, date of culture, antimicrobial susceptibilities, and patient demographic data associated with each strain were retrieved from the electronic medical records under an approved protocol of the institutional review board of the University of Alabama at Birmingham with a waiver of informed consent. Noninvasive pneumococci for this study were defined as *S. pneumoniae* strains causing infection detected in ear, eye, nasopharynx, or tracheal aspirate specimens and in which no invasive (sterile site) isolates were collected from the same patient. Nasopharyngeal isolates were not collected as part of a screen for normal carriage flora but from surgical samples and patients being screened for pathogens in their nasopharynx as part of routine medical care. A total of 1,130 noninvasive strains were collected; 72 strains were excluded because they were duplicates of the same capsular type from the same patient, similar to another isolate from the same clinical episode, not viable, or the patient was >18 years old. The analyses included 1,058 noninvasive isolates and 157 IPD isolates characterized previously (6).

Multiplex assays for serotype detection. The strains were typed serologically and/or by PCR for the 93 known pneumococcal capsular types. The strains were first subjected to a multiplex immunoassay with monoclonal antibodies that were specific for 26 serotypes as described by Yu et al. (46). Isolates that were not typeable by the multiplex immunoassay were further typed using a multiplex PCR assay for the remaining serotypes, which also included the detection of autolysin (*lytA*), two different primer sets for the detection of *cpsA*, and the nontypeable groups NCC2 and NCC3 (47) of *S. pneumoniae*. For analyses, strains were grouped according to whether their capsular serotype was included or not included as an antigen in: (i) the original heptavalent PCV (PCV7), (ii) the capsule antigens for the 7 serotypes in PCV7 plus six additional serotypes (PCV13), (iii) strains with capsule types not included in PCV13 but including isolates with typeable capsules (NVT), and (iv) those with nontypeable capsules (NT).

NT strains were confirmed to be from *S. pneumoniae* through testing for optochin sensitivity, bile solubility, and presence of the pneumolysin gene (*ply*) (Table 1) (48). Strains that were bile insoluble and not positive for *ply* were removed from analyses.

Gene typing. Gene typing was performed on all IPD isolates and a random sample of the noninvasive strains (strains selected by using the random number generator function on Microsoft Office Excel [Microsoft Redmond Campus, Redmond, WA]). Genomic DNA was prepared using a modified protocol with the Easy-DNA kit (Life Technologies, Carlsbad, CA). Briefly, the strains were streaked from frozen glycerol stock onto blood agar plates and incubated overnight in a candle jar in a 37°C incubator. The next day, the entire plate was swabbed into 3 ml of Todd-Hewitt medium with 5% yeast and grown for 4 to 6 h. DNA was then isolated according to the manufacturer's instructions, the concentration was determined by the NanoDrop 2000 (Thermo Scientific, Waltham, MA), and DNA was diluted to a final concentration of 100 ng/μl for further use.

PCRs were conducted as previously described using the primer pairs listed in Table 1 (45, 49, 50). All strains were typed at least twice for each gene. The reference strains *S. pneumoniae* TIGR4 (from serotype 4) and EF3030 (from serotype 19F) were used as controls.

Statistical analysis. The statistical analyses between proportions of groups were performed using the χ^2 test, the χ^2 test for trend, or Fisher's exact test. Bonferroni's correction was applied when necessary. A P value of <0.05 was considered statistically significant.

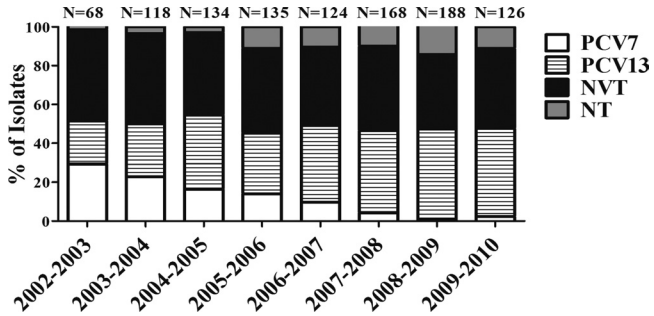

FIG 1 Serotype distribution of noninvasive isolates by period of isolation. Serotype distributions for IPD isolates are shown for the years 2002 through 2010. Each seasonal period spans from July 1 through June 30 of the second year. *N*, total number of isolates from the indicated period; PCV7, capsular serotypes in the heptavalent pneumococcal conjugate vaccine; PCV13, capsular serotypes in the 13-valent PCV that are not included in PCV7; NVT, typeable serotypes not included in the PCV13; NT, nontypeable isolates. Overall, 49% of noninvasive infections were caused by serotypes included in the Prevnar13 (PCV13).

TABLE 2 Serotype distribution by site of isolation for noninvasive disease isolates

PCV group and serotype	No. (%) of noninvasive strains from each isolation site[a]					Total no. of strains (% of total)
	Ear	Eye	Np	Trach	Other	
PCV7						
19F	49 (7.2)	1 (1.2)	8 (8.6)	14 (8.3)	3 (8.3)	75 (7.1)
6B	3 (0.44)	3 (3.6)		6 (3.6)	1 (2.8)	13 (1.2)
23F	4 (0.59)			6 (3.6)	2 (5.6)	12 (1.1)
9V	1 (0.15)	1 (1.2)		2 (1.2)		4 (0.38)
18C	2 (0.30)		1 (1.1)			3 (0.28)
4	1 (0.15)			1 (0.60)		2 (0.19)
14	2 (0.30)					2 (0.19)
PCV13						
19A	241 (35.6)	24 (28.6)	29 (31.2)	34 (20.2)	10 (27.8)	338 (31.9)
3	20 (3.0)	3 (3.6)	2 (2.2)	4 (2.4)		29 (2.7)
6A	15 (2.2)	3 (3.6)	2 (2.2)	5 (3.0)	1 (2.8)	26 (2.5)
7F	6 (0.89)	1 (1.2)	1 (1.1)		1 (2.8)	9 (0.85)
5		1 (1.2)				1 (0.09)
Non-PCV						
NT[b]	58 (8.6)	12 (14.3)	13 (14.0)	24 (14.3)	4 (11.1)	111 (10.5)
35B	66 (9.7)	11 (13.1)	6 (6.5)	12 (7.1)	2 (5.6)	97 (9.3)
6C	36 (5.3)	5 (6.0)	11 (11.8)	12 (7.1)	4 (11.1)	68 (6.4)
15B/C	41 (6.1)	2 (2.4)	3 (3.2)	9 (5.4)	4 (11.1)	59 (5.6)
11A/D/F	21 (3.1)	5 (6.0)	4 (4.3)	8 (4.8)		38 (3.6)
33F/A	23 (3.4)		1 (1.1)	5 (3.0)		29 (2.7)
22F/A	13 (1.9)	4 (4.8)	2 (2.2)	7 (4.3)	1 (2.8)	27 (2.6)
15A/F	19 (2.8)	1 (1.2)	1 (1.1)	2 (1.2)		23 (2.2)
16	18 (2.7)		2 (2.2)	2 (1.2)		22 (2.1)
23B	7 (1.0)		2 (2.2)	3 (1.8)		12 (1.1)
23A	5 (0.74)	2 (2.4)	1 (1.1)		1 (2.8)	9 (0.85)
9N	3 (0.44)	1 (1.2)	1 (1.1)	3 (1.8)		8 (0.76)
35F/47F	4 (0.59)	2 (2.4)		1 (0.60)	1 (2.8)	8 (0.76)
7B/C/40	5 (0.74)			2 (1.2)		7 (0.66)
17F/A	4 (0.59)			3 (1.8)		7 (0.66)
31	3 (0.44)	2 (2.4)		1 (0.60)		6 (0.57)
34	2 (0.30)		1 (1.1)	1 (0.60)		4 (0.38)
10A/39	1 (0.15)			1 (0.60)	1 (2.8)	3 (0.28)
21	2 (0.30)		1 (1.1)			3 (0.28)
12F/B	1 (0.15)		1 (1.1)			2 (0.19)
11E	1 (0.15)					1 (0.09)
Total	677 (64)	84 (7.9)	93 (8.8)	168 (15.9)	36 (3.4)	1,058 (100)

[a] Np, nasopharyngeal; Trach, tracheal aspirate; other, isolates from sinus tissue, nasal lacrimal duct, or mastoid.
[b] NT, nontypeable (capsular serotype could not be determined).

To determine the probabilities of invasive disease potential in the serotypes, odds ratios (ORs) were calculated as $(ad)/(bc)$, where a represents the number of invasive A serotypes, b represents the number of noninvasive A serotypes, c represents the number of invasive non-A serotypes, and d represents the number of noninvasive non-A serotypes or clones (51). An OR of >1 indicates increased invasive potential, whereas an OR of <1 indicates decreased invasive potential. An OR was considered statistically significant at a P value of <0.05. All tests were performed in GraphPad InStat version 5.0 (GraphPad, La Jolla, CA).

RESULTS

Patient demographics and serotype distribution of isolates from 2002 to 2010. The population was 60% male, 63% Caucasian, and 14% African-American (data on race/ethnicity were missing for 18% of patients). Of 1,055 IPD isolates for which the patient age was available, 675 (64%) isolates were obtained from children <24 months of age, 282 (27%) isolates were from children aged 24 to 60 months, and 98 (9%) isolates were from children >60 months. In order to capture the seasonality of pneumococcal illness, the data were grouped by year, with the year being defined from July 1 through June 30. Thirty-three serotypes, including the nontypeable serotypes, were identified in the noninvasive strains. The PCV7 capsular types comprised approximately 29% of the noninvasive strains in 2002 and had virtually disappeared by 2010 (2002 to 2008 versus 2008 to 2010, $P < 0.0001$). There was no statistically significant difference between patient age and PCV13 versus non-PCV13 types ($P = 0.3627$, data not shown). The proportion of non-PCV13 capsular types also remained steady at approximately 50% from 2002 to 2010. A total of 21 different non-PCV13 capsular types were observed among the strains shown in Fig. 1. Serotype 19A was the predominant serotype isolated from noninvasive strains (32%), followed by 35B (9%), 19F (7%), and 6C (6%) (Table 2). The majority of the noninvasive isolates came from the ear (64%), and

TABLE 3 Invasive potential of *S. pneumoniae* isolates

PCV group and serotype[a]	No. of strains	No. of isolates by type		OR (95% CI)[b]
		IPD	Noninvasive	
PCV7				
14	5	3	2	**10.3 (1.7–62.1)**
4	3	1	2	3.4 (0.3–37.6)
18C	3	1	2	3.4 (0.3–37.6)
9V	5	1	4	1.7 (0.2–15.2)
23F	14	2	12	1.1 (0.2–5.1)
6B	15	2	13	1.0 (0.2–4.6)
19F	85	9	76	0.8 (0.4–1.6)
PCV13				
5	3	2	1	**13.7 (1.2–151.5)**
7F	22	13	9	**10.5 (4.4–25.1)**
3	35	6	29	1.4 (0.6–3.5)
19A	389	51	338	1.0 (0.7–1.5)
6A	27	1	26	0.3 (0.03–1.9)
1	3	3	0	
Non-PCV				
12F/B	8	6	2	**21.0 (4.2–105.0)**
11E	2	1	1	6.8 (0.4–109.0)
10A/39	5	2	3	4.5 (0.8–27.4)
17F/A	11	4	7	**3.9 (1.1–13.6)**
23A	13	4	9	3.0 (0.9–10.0)
23B	16	4	12	2.3 (0.7–7.2)
22F/A	33	6	27	1.5 (0.6–3.7)
15B/C	67	8	59	0.9 (0.4–1.9)
9N	9	1	8	0.8 (0.1–6.8)
35F/47F	9	1	8	0.8 (0.1–6.8)
6C	75	7	68	0.7 (0.3–1.5)
15A/F	25	2	23	0.6 (0.1–2.5)
16F	24	2	22	0.6 (0.1–2.6)
33F/A	31	2	29	0.5 (0.1–1.9)
35B	103	6	97	**0.4 (0.2–0.9)**
11A/D/F	39	1	38	0.2 (0.02–1.3)
NT	115	4	111	**0.2 (0.1–0.6)**
7B/C/40	7	0	7	
31	6	0	6	
34	4	0	4	
21	3	0	3	
13	1	1	0	
Total	1,215	157	1,058	

[a] NT, nontypeable. Each serotype group is ordered by invasive potential.
[b] OR, odds ratio; CI, confidence interval. An OR of >1 indicates increased invasive potential, whereas an OR of <1 indicates decreased invasive potential. ORs (95% CI) are considered statistically significant at a *P* value of <0.05 (shown in bold type).

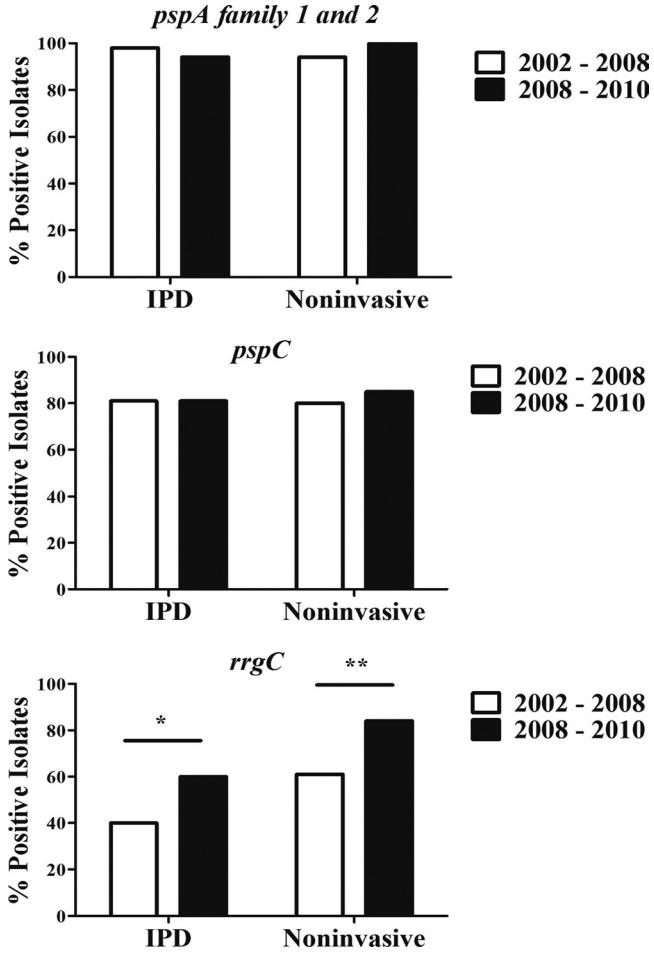

FIG 3 Gene frequencies over a period of time in IPD and noninvasive isolates. IPD ($n = 157$) and noninvasive ($n = 221$) isolates were typed by PCR for *pspA*, *pspC*, or *rrgC* and grouped into two periods, 2002 to 2008 and 2008 to 2010. *, $P < 0.05$; **, $P < 0.001$. *P* values were obtained using Fisher's exact test.

the serotype distribution of the noninvasive isolates varied depending on the site of isolation. However, this difference was not statistically significant (PCV13 versus non-PCV13 types, $P = 0.3205$). Compared with IPD serotype distribution, serotypes 14, 5, 7F, 12F/B, and 17F/A were found to have more invasive potential, whereas serotype 35B and NT strains were associated with lower invasiveness (Table 3).

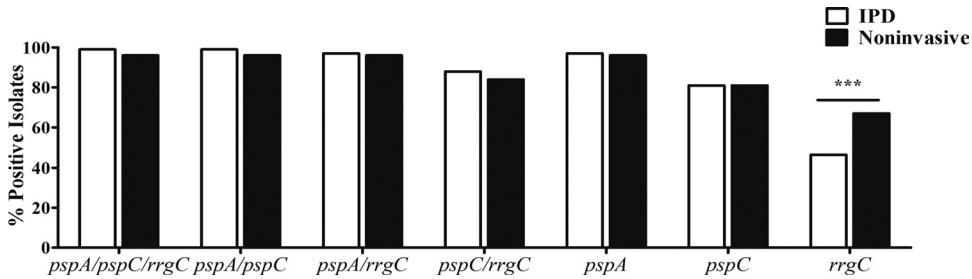

FIG 2 Gene frequencies in IPD and noninvasive isolates. IPD ($n = 157$) and noninvasive ($n = 221$) isolates were typed by PCR for *pspA*, *pspC*, or *rrgC*. Gene names separated by slashes refer to pneumococci that are positive for those genes. All strains were typed at least twice for each gene. ***, $P < 0.0001$. *P* values were obtained using Fisher's exact test.

TABLE 4 Serotype distribution and gene frequency by disease group

Serotype[a]	No. (%) of strains with genes by disease group									
	IPD					Noninvasive				
	In the presence of:					In the presence of:				
	pspA family				Total for serotype	*pspA* family				Total for serotype
	1	2	*pspC*	*rrgC*		1	2	*pspC*	*rrgC*	
4		1 (100)	1 (100)	1 (100)	1					
6B	2 (100)		2 (100)	1 (50)	2		2 (100)	2 (100)	2 (100)	2
9V		1 (100)	1 (100)	1 (100)	1					
14	1 (33)	2 (67)	3 (100)		3					
18C	1 (100)		1 (100)		1					
19F	1 (11)	8 (89)	7 (78)	9 (100)	9	2 (20)	8 (80)	10 (100)	9 (90)	10
23F	2 (100)		2 (100)	1 (50)	2	2 (100)		1 (50)	1 (50)	2
1	3 (100)		3 (100)	1 (33)	3					
3	2 (33)	4 (67)	4 (67)	2 (33)	6	1 (50)	1 (50)	1 (50)	1 (50)	2
5	2 (100)		2 (100)	1 (50)	2					
6A	1 (100)		1 (100)		1	2 (67)	1 (33)	2 (67)	2 (67)	3
7F[b]	1 (8)	11 (85)	13 (100)	2 (15)	13					
19A[b]	17 (33)	33 (65)	44 (86)	37 (73)	51	20 (36)	36 (64)	55 (98)	46 (82)	56
6C	7 (100)		5 (71)	2 (29)	7	12 (100)		11 (92)	9 (75)	12
9N	1 (100)		1 (100)		1					
10A/39	2 (100)		2 (100)		2	1 (100)		1 (100)	1 (100)	1
11A/D/F		1 (100)			1	1 (17)	5 (83)	4 (67)	4 (67)	6
11E		1 (100)	1 (100)		1					
12F/B		6 (100)	5 (83)	1 (17)	6		1 (100)	1 (100)	1 (100)	1
13		1 (100)		1 (100)	1					
15A/F		2 (100)		1 (50)	2	1 (17)	5 (83)	3 (50)	2 (33)	6
15B/C	2 (25)	6 (75)	6 (75)	1 (12.5)	8		10 (100)	9 (90)	7 (70)	10
16F	1 (50)	1 (50)	2 (100)		2	2 (40)	3 (60)	5 (100)	4 (80)	5
17F/A		4 (100)	3 (75)	1 (25)	4		1 (100)	1 (100)	1 (100)	1
21							1 (100)	1 (100)		1
22F/A[b]	6 (100)		3 (50)	1 (17)	6	2 (50)	1 (25)	2 (50)	1 (25)	4
23A	4 (100)		4 (100)	2 (50)	4	1 (100)		1 (100)		1
23B	2 (50)	2 (50)	3 (75)	1 (25)	4	1 (33)	2 (67)	1 (33)	2 (67)	3
31						2 (100)		2 (100)	1 (50)	2
33F/A	2 (100)		2 (100)		2	4 (80)	1 (20)	5 (100)	1 (20)	5
35B		6 (100)	5 (83)	6 (100)	6		19 (100)	15 (79)	19 (100)	19
35F/47F		1 (100)			1		4 (100)	2 (50)	1 (25)	4
NT[b]		1 (25)	1 (25)		4	12 (18)	45 (69)	45 (69)	34 (52)	65
Total	60 (38)	92 (59)	127 (81)	73 (46)	157	66 (30)	146 (66)	180 (81)	149 (67)	221

[a] NT, nontypeable.
[b] These serotypes had 1 strain that was nontypeable for *pspA*. NT isolates had >1 strain that was nontypeable for *pspA*.

Characterization of isolates for *pspA*, *pspC*, and *rrgC* alleles. All the IPD isolates ($n = 157$) and a subset of the noninvasive isolates ($n = 221$) were selected for genetic characterization of pneumococcal protein candidate antigens PspA, PspC, and RrgC (pilus type 1). Eleven isolates (2 IPD, 9 noninvasive) were negative for all three genes. Although the *pspA* and *pspC* genes are highly mosaic (24, 52), the majority of isolates were *pspA* family 1 or 2 types (97%) and carried *pspC* alleles (81%), regardless of the disease group (Fig. 2). We also looked at the frequency of *rrgC*, a highly conserved gene in strains carrying pilus type 1 (44, 45). We found a high frequency of *rrgC*-positive isolates in both the IPD and noninvasive strains (46 and 67%, respectively). Furthermore, noninvasive isolates (67%) were significantly more likely to be *rrgC* positive than IPD strains (47%) ($P < 0.0001$). When we looked at how the strains changed from 2002 to 2008 versus 2008 to 2010, we saw a nonsignificant increase in *pspA* (family 1 or 2) in the noninvasive isolates and an increase in *rrgC*-positive strains in both the IPD and noninvasive groups (40% versus 60% for IPD strains, $P = 0.02$; 61% versus 84% for noninvasive strains, $P < 0.001$) (Fig. 3). The frequency of *pspC* remained constant at 81%. Lastly, no association was seen between *pspA* or *pspC* positivity and serotype (Table 4). Although the PCV13 types were significantly more likely to be *rrgC* positive than the NVT strains ($P < 0.001$), 50% of the NVT strains were *rrgC* positive overall (Fig. 4). Three serotypes, 19F, 19A, and 35B, were significantly associated with being *rrgC* positive (78, 95, and 100%, respectively; $P < 0.005$).

DISCUSSION

In this study, we report the capsular serotype distribution and gene frequencies of specific pneumococcal virulent proteins collected from 2002 to 2010 in noninvasive and IPD isolates to esti-

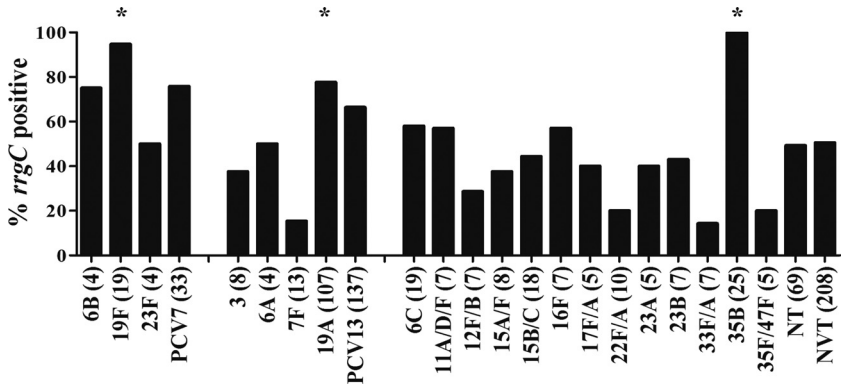

FIG 4 Strains of PCV13 serotypes are more likely to be *rrgC* positive. All strains were grouped together by serotype, regardless of disease group, and *rrgC* frequency was determined. Only serotypes with 4 isolates or more are shown; however, all strains were included in determining the proportion expressing *rrgC* in the PCV7, PCV13, and NVT groups, even if a serotype is not represented in the graph. The number of isolates per serotype is given in parentheses. *, $P < 0.005$, per Fisher's exact test with Bonferroni's correction.

mate the potential coverage of the PCV13. In our collection of 1,058 noninvasive strains, only 50% of all strains were PCV13 types. This was consistent from year to year, regardless of the site of isolation (data not shown). Twenty-one capsular serotypes were not covered by the PCV13, and this raises the possibility that some of them will evolve over time to become major replacement strains. Because of this, serotype replacement seems very likely considering the number of different capsular types that will have the chance to acquire the needed genes to effectively replace the PCV strains. Preclinical trials are in progress for a new conjugate vaccine, the PCV15 (53), but it will still lack coverage for 21 capsular types presently found in Alabama children and adolescents, some of which are considered to have high invasive potential (Table 3, 17F/A and 12F/B).

To this end, we also characterized the gene frequencies of several pneumococcal virulence factors, *pspA*, *pspC*, and *rrgC*, to determine the potential coverage of their proteins. While previous studies have looked at *pspA* frequencies (54–58), those studies were focused on pre-PCV7 strains, which are no longer representative of the serotypes currently isolated in the United States, or strains from nonpediatric populations (49). In a recent report, we showed that IPD strains collected in Alabama from 2002 to 2010 were of *pspA* family 1 or family 2 in 96% of cases (6). Since the majority of strains collected from patients over the same period were not from invasive sites, it was important to look at this larger group of strains since it was possible that they might provide a window into strains that cause IPD in the future. Moreover, not much is known about the epidemiology of noninvasive strains. We found that almost all noninvasive pneumococci tested contained *pspA* family 1 or 2 alleles (~97%, $n = 221$), with *pspA* family 2 being the more common allele. Moreover, these distributions closely reflected that seen within the IPD collection. We also looked at the frequency of *pspC*. Similar to PspA, PspC is also highly variable (52); however, in our collection of strains, we found that 81% of all pneumococci were positive for *pspC*, regardless of the disease or *S. pneumoniae* serotype.

We also looked at the frequency of *rrgC*, a highly conserved subunit of pilus type 1 (44, 45). We found that the noninvasive isolates were significantly more likely to be *rrgC* positive ($P < 0.0001$). This makes sense because pili act as adhesins, binding to epithelial cells (41, 42). Although pili may be important for colonization, 46% of IPD isolates were *rrgC* positive. We also saw an association between *rrgC* and serotype, similar to previously reported data (44, 45, 59) where PCV13 vaccine types were more likely to be *rrgC* positive ($P < 0.001$). In our collection, serotypes 19A, 19F, and 35B in particular were highly *rrgC* positive ($P < 0.005$). However, contrary to the previous studies, we saw a significant increase in *rrgC*-positive strains during the period of 2008 to 2010 compared to the period from 2002 to 2008 (Fig. 3). Since many of the original PCV7 strains express *rrgC*, it is possible that noninvasive non-PCV7 strains with *rrgC* are favored by selection because they can better fill the old PCV7 niche.

This study has some limitations, in that we only looked at isolates from a single geographic region and a random sampling of noninvasive strains for the presence of genes for protein virulence factors. However, this random sample represents the overall serotype distribution very well, and the gene frequencies were similar to those of the IPD isolates. Another limitation is that we report gene frequencies and do not know whether the associated proteins are produced and/or functional in these pneumococci.

In conclusion, the serotype and gene-type distributions were remarkably similar for IPD and noninvasive strains from pediatric patients. Our Alabama strain collection contained 21 non-PCV13 serotypes that may evolve to fill the niche left following PCV usage. More importantly, these serotypes have been shown to cause life-threatening meningitis and endocarditis (6, 8). Based on our collection of isolates, the virulence proteins PspA, PspC, and RrgC have the potential to cover a wider number of strains than the PCV13 and PCV15 vaccines, although the efficacy of these proteins as vaccines in humans is still not known. The inclusion of additional proteins with PspA may not add coverage but may provide greater protective efficacy, since previous studies in mice have shown higher vaccine efficacy with mixtures of protein antigens rather than single proteins.

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D.E.B. is a consultant for Sanofi Pasteur and the PATH Foundation. The University of Alabama at Birmingham (UAB) holds intellectual property rights related to protein vaccine antigens, including PspA, and D.E.B. is an inventor on the relevant patents. UAB also holds the intellectual property rights for monoclonal antibodies used in this study. C.M.C., M.H.N., D.E.B., and M.J.C. are employees of the UAB.

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