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Noninvasive Pneumococcal Clones Associated with Antimicrobial Nonsusceptibility Isolated from Children in the Era of Conjugate Vaccines

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Carriage and noninvasive pneumococcal isolates frequently have a higher prevalence of antimicrobial nonsusceptibility than invasive isolates. From 2009 to 2014, we determined the associated clones in 169 pediatric noninvasive nonsusceptible pneumococci from a total of 506 isolates collected after 7- and 13-valent conjugate vaccine introduction (PCV7/13) to the Irish childhood immunization schedule in 2008 and 2010, respectively. We compared our results to those from 25 noninvasive pediatric pneumococcal isolates collected in 2007, the year before introduction of conjugate vaccines. In 2007, England^{14}-9 and Spain^{9V}-3 accounted for 12% and 32% of nonsusceptible clones, respectively, but in 2009 to 2014, their prevalence fell to 0% and 2.4%. Furthermore, there was a significant decline in Spain^{6B}-2 and its variants from 2009 to 2014 ($P = 0.0024$). Fluctuations occurred in clonal complex 320 associated with serotype 19A. The prevalence of Sweden^{15A}-25 and its variants and ST558 (a single-locus variant of Utah^{35B}-24) associated with nonvaccine serotypes (NVT) 15A and 35B increased from 0% and 8% in 2007 to 19% and 16% in 2013 to 2014, respectively. Pilus locus 1 (PI-1) is associated with the spread of some nonsusceptible pneumococcal clones. PI-1 was more frequently associated with PCV7/13 serotypes than NVT ($P = 0.0020$). Our data highlight the value of surveillance of noninvasive pneumococci following conjugate vaccine introduction. Importantly, emerging clones associated with NVT may limit the effectiveness of PCV7/13 in reducing the high rate of nonsusceptibility among pediatric noninvasive pneumococci, with implications for empirical treatment strategies.

Streptococcus pneumoniae causes invasive infections, such as meningitis, or noninvasive infections, including conjunctivitis and otitis media. The organism is frequently carried asymptomatically in the nasopharynx of young children and can be transmitted to adults (1). Antimicrobial-nonsusceptible pneumococci pose a challenge for devising effective empirical antimicrobial treatment strategies.

Pneumococci are encapsulated with 95 serotypes described. Nontypeable (NT) pneumococci lack a capsule. Pneumococci of the same serotype may have genetically distinct backgrounds (i.e., clones), and multilocus sequence typing (MLST) characterizes pneumococci into related clonal groups or sequence types (STs) (2). Antimicrobial nonsusceptibility is common among some pneumococcal clones, such as Spain^{6B}-2, England^{14}-9, Sweden^{15A}-25, and clonal complex 320 (CC320) (3). The presence of a pilus (pilus locus 1 [PI-1]) among some nonsusceptible clones may contribute to their spread (4, 5).

The first pneumococcal conjugate vaccine was the 7-valent pneumococcal conjugate vaccine (PCV7), targeting serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. These serotypes were previously important in carriage and disease (6). Importantly, PCV7 also targeted several serotypes belonging to antimicrobial-nonsusceptible clones (3). Following PCV7 introduction, non-PCV7 serotypes increased, including serotype 19A (7–11). The frequency of serotype 19A is of concern as many 19A-associated clones, such as CC320, are antimicrobial nonsusceptible (3). The 13-valent pneumococcal conjugate vaccine (PCV13) targeting PCV7 serotypes plus serotypes 1, 3, 5, 6A, 7F, and 19A is now used in several countries and should further impact positively on nonsusceptible pneumococci. Surveillance of pneumococcal serotypes/clones following PCV13 introduction will determine if antimicrobial-nonsusceptible clones expressing nonvaccine serotypes (NVT) emerge. This surveillance should encompass pediatric carriage and noninvasive infections, as greater rates of nonsusceptibility are reported in these isolates (12).

PCV7 was introduced to the Irish childhood immunization schedule in 2008 and replaced by PCV13 in 2010. Previously, we reported on pneumococcal serotypes associated with nonsusceptibility in an Irish pediatric hospital (9). In the present study, we used MLST to further characterize these nonsusceptible noninvasive pneumococci. We also included nonsusceptible pneumococcal isolates from two other Irish pediatric hospitals collected in 2013 to 2014. We determined the prevalence of PI-1 among these isolates, given its association with some nonsusceptible clones (4, 5). Finally, we compared our results to those from a collection of

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noninvasive antimicrobial-nonsusceptible pneumococci collected before PCV7 introduction.

(Data on serotypes and associated nonsusceptibility patterns from 2007 and 2009 to 2012 were reported in reference 9. Nonsusceptible serotypes from 2013 were presented in part at the 32nd Annual Meeting of the European Society for Pediatric Infectious Diseases [35]. Sequence type data from 2009 to 2011 were presented at the 23rd European Congress for Clinical Microbiology and Infectious Diseases, Berlin, 2013 [36].)

MATERIALS AND METHODS

Isolate collection. Pneumococci were isolated from carriage and noninvasive infections (i.e., conjunctivitis, nonbacteremic lower respiratory tract infection, and otitis media) at Temple Street Children's University Hospital from January to December 2007 and January 2009 to December 2012 as previously described (9). The serotype data and antimicrobial susceptibilities of isolates from 2009 to 2012 were presented previously (9). From the first quarter of 2013 to the first quarter of 2014, carriage and noninvasive pneumococci isolated at Temple Street Children's University Hospital and two other Irish pediatric hospitals in Dublin, Our Lady's Children's Hospital, Crumlin, and the Adelaide and Meath Hospital, Tallaght, were collected. Just one isolate from each child was included in the study. All hospitals provide pediatric secondary care to catchment localities (population of approximately 1.3 million) and national tertiary referral services. Children from whom pneumococci were isolated ranged in age from 10 days to 16 years. Carriage isolates included nasopharyngeal swabs and aspirates and nasal and throat swabs. Ethical approval was granted by the Ethics Committee at Temple Street Children's University Hospital.

Serotyping and antimicrobial susceptibility testing. Serotyping was performed using multiplex PCR and the standard capsular reaction using antisera (Statens Serum Institut, Copenhagen, Denmark) (9, 13). Multiplex PCR detected serotypes 1, 3, 4, 5, 6, 7F/a, 9V/a, 14, 18, 19F, and 23F. The standard capsular reaction confirmed serotypes identified by PCR and identified serotypes not detected by PCR. Susceptibilities to penicillin, cefotaxime, tetracycline, erythromycin, clindamycin, and levofloxacin were determined using Etest (bioMérieux, Marcy l'Etoile, France). Results were interpreted using the 2013 Clinical and Laboratory Standards Institute guidelines (14). Oral and meningitis breakpoints were used to define susceptibilities to penicillin and cefotaxime, respectively (14). Nonsusceptible isolates were defined as those displaying intermediate or complete resistance to one or more of the six antimicrobials tested for. Multidrug-resistant isolates were those with complete resistance to three or more antimicrobials.

DNA extraction. DNA was purified from overnight cultures grown on 5% sheep blood by either a manual method (GentraPurgene Yeast/Bact kit; Qiagen, N.V., Venlo, The Netherlands) or an automated method (QIAcube HT kit; Qiagen, N.V., Venlo, The Netherlands).

MLST. All nonsusceptible pneumococci were subjected to MLST, which was performed by standard procedures (2). Allele numbers and sequence types (STs) were assigned using http://pubmlst.org/. eBURST was used to assign clonal complexes (CCs) (15). Sequence types (STs) were compared with the Pneumococcal Molecular Epidemiology Network (PMEN) clones at http://www.sph.emory.edu/PMEN.

Detection of PI-1. Nonsusceptible pneumococci were screened for PI-1. Primers targeting the *rlrA* gene were used to detect PI-1 (16). This gene encodes the RlrA protein, necessary for expression of structural genes of PI-1 (17). *S. pneumoniae* strains TIGR4 and TIGR6 were used as positive and negative controls, respectively.

Statistical analysis. Proportions were compared using the two-tailed Fisher's exact test on the GraphPadQuickCalcs website http://www.graphpad.com/quickcalcs/contingency1/ (accessed December 2014). A P value of ≤ 0.05 was considered significant. Simpson's index of diversity (D)—defined as $D = \Sigma\, n(n-1)/N(N-1)$, where N = total number of isolates and n = total number of isolates of a particular ST—was used to calculate the diversity of STs associated with nonsusceptibility for each year of the study and within selected nonsusceptible serotypes (18). The Shannon index—defined as $\Sigma\, fi \log_2 fi$, where fi is the frequency of individual STs—was used to calculate the evenness of STs distributed among nonsusceptible pneumococci each year (19).

TABLE 1 Percentage of carriage and noninvasive pneumococcal isolates nonsusceptible to penicillin, cefotaxime, tetracycline, erythromycin, clindamycin, and levofloxacin in Irish pediatric hospitals from 2009 to 2014

Antimicrobial	% of isolates:	
	Resistant	Intermediate
PEN	6.5	21
CTX	1	8
TET	20	0.6
ERY	25	0
CLI	19	0.6
LVX	0.2	0

[a] PEN, penicillin; CTX, cefotaxime; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; LVX, levofloxacin.

RESULTS

Prevalence of antimicrobial-nonsusceptible isolates. The total number of pneumococci collected in 2007 and 2009 to 2014 was $n = 611$. The distribution of isolates each year was as follows: 2007, $n = 105$; 2009, $n = 46$; 2010, $n = 121$; 2011, $n = 89$; 2012, $n = 83$; and 2013 to 2014, $n = 167$. The numbers of pneumococci isolated from each anatomical site from 2007, 2009, 2010, 2011, 2012, and 2013 to 2014, respectively, were as follows: carriage, $n = 44$, 18, 48, 39, 30, and 41; conjunctivitis, $n = 24$, 10, 30, 23, 21, and 75; nonbacteremic lower respiratory tract infections, $n = 26$, 12, 28, 17, 21, and 30; and otitis media, $n = 11$, 6, 15, 10, 11, and 21. From 2009 to 2012, most carriage isolates were collected preoperatively from children undergoing upper respiratory surgery. Other carriage isolates collected during this period and 2013 to 2014 were from dermatology clinics, the emergency department, and general practitioners. The numbers of pneumococci nonsusceptible to at least one antimicrobial each year were as follows: 2007, $n = 25$ (23%); 2009, $n = 14$ (30%); 2010, $n = 38$ (31%); 2011, $n = 19$ (21%); 2012, $n = 29$ (35%); and 2013 to 2014, $n = 69$ (41%). The percentage of pneumococci resistant and intermediate to each antimicrobial tested for from 2009 to 2014 is shown in Table 1. The mean age of children overall was 36 months—37 months in children from whom antimicrobial-nonsusceptible pneumococci were isolated. Nonsusceptibility rates among carriage, conjunctivitis, nonbacteremic lower respiratory tract infection, and otitis media from 2009 to 2014 were 29, 28, 43, and 44%, respectively.

Antimicrobial-nonsusceptible serotypes and clones from 2009 to 2014. From 2009 to 2014, nonsusceptibility occurred among 15 serotypes, nontypeable pneumococci, and 60 different STs (Table 2). Of the 60 STs, 29 ($n = 104$ isolates) were Pneumococcal Molecular Epidemiology Network (PMEN) clones and/or single- or double-locus variants (SLV or DLV, respectively). The most common nonsusceptible STs and associated serotypes are outlined in Table 2.

Patterns of nonsusceptibility among serotypes and clones from 2009 to 2014. Sweden^{15A}-25, Spain^{6B}-2, and CC320 and

TABLE 2 Serotypes, STs, CCs or PMEN clones, and associated nonsusceptibilities to six different antimicrobials among carriage and noninvasive pneumococci isolated in Irish pediatric hospitals from 2009 to 2014

Serotype	ST (no. of isolates)	CC or PMEN clone	Nonsusceptibility pattern (no. of isolates)[a]					
			PEN	CTX	TET	ERY	CLI	LVX
19A[b]	320 (14)	CC320	R (12), I (2)	R, (4), I (10)	R (14)	R (14)	R (14)	S (14)
	63 (11)	Sweden^{15A}-25	I (11)	S (11)	R (11)	R (11)	R (11)	S (11)
	276 (3)	SLV Denmark^{14}-32	I (3)	S (3)	R (3)	R (3)	R (3)	S (3)
	Other (3)		I (3)	I (1), S (2)	S (2), R (1)	S (2), R (1)	S (2), R (1)	S (3)
6B[b]	94 (10)	SLV Spain^{6B}-2	R (5), I (5)	I (7) S (3)	R (10)	R (10)	R (10)	S (10)
	90 (5)	Spain^{6B}-2	R (1), I (4)	I (4), S (1)	R (4), S (1)	R (5)	R (5)	S (5)
	386 (2)	DLV Poland^{6B}-20	S (2)	S (2)	R (2)	R (2)	R (2)	S (2)
	9006 (3)	SLV Poland^{6B}-20	I (3)	S (3)	R (3)	R (3)	R (3)	S (3)
	Other (4)		I (2), S (2)	S (4)	R (2), S (2)	R (4)	R (2), S (2)	R (1), S (3)
35B	558 (21)	SLV Utah^{35B}-24	I (21)	I (2), S (19)	S (21)	R (2), S (19)	S (21)	S (21)
	7491 (1)	DLV Utah^{35B}-24	I (1)	S (1)	S (1)	R (1)	S (1)	S (1)
15A	63 (6)	Sweden^{15A}-25	I (6)	S (6)	R (2), S (4)	R (6)	R (6)	S (6)
	374 (5)	SLV Sweden^{15A}-25	I (5)	S (5)	R (5)	R (5)	R (5)	S (5)
	Other (10)		R (3), I (7)	R (1), I (1), S (8)	R (7), S (3)	R (8), S (2)	R (7), S (3)	S (10)
19F[b]	2100 (4)	SLV Sweden^{15A}-25	R (2), I (2)	I (2),S (2)	R (4)	R (4)	R (4)	S (4)
	87 (2)		I (2)	S (2)	R (1), S (1)	R (2)	R (2)	S (2)
	Other (9)		R (2), I (4), S (3)	I (3), R (1), S (5)	R (9)	R (9)	R (7), S (2)	S (9)
NT	4149 (4)	SLV Norway^{NT}-42	R (1), I (3)	I (3), S (1)	R (4)	R (4)	R (3), S (1)	S (4)
	344 (3)	Norway^{NT}-42	I (2), S (1)	S (3)	R (2), S (1)	R (2), S (1)	R (1), S (2)	S (3)
	Other (8)		R (2), I (4), S (2)	I (2) S (6)	R (6), S (2)	R (6), S (2)	R (1), S (7)	S (8)
33F	717 (4)		S (4)	S (4)	R (1), I (3)	R (4)	R (4)	S (4)
	100 (2)		S (2)	S (2)	S (S)	R (2)	S (2)	S (2)
	Other (2)		S (2)	S (2)	S (2)	R (2)	R (2)	S (2)
23B	2372 (3)		I (3)	S (3)	S (3)	S (3)	S (3)	S (3)
	439 (2)	SLV Tennessee^{23F}-4	S (2)	S (2)	S (2)	R (2)	S (2)	S (2)
	Other (2)		I (2)	S (2)	S (2)	S (2)	I (1), S (1)	S (2)
6A[b]	473 (6)		I (5), S (1)	S (6)	S (6)	R (6)	S (6)	S (6)
	65 (1)		S (1)	S (1)	S (1)	R (1)	S (1)	S (1)
15B/C	199 (3)	Netherlands^{15B}-37	S (3)	S (3)	S (3)	R (3)	S (3)	S (3)
	7479 (3)		I (3)	S (3)	R (3)	R (3)	I (2), S (1)	S (3)
23F[b]	81 (2)	Spain^{23F}-1	R (2)	I (2)	R (2)	R (1), S (1)	R (1), S (1)	S (2)
	Other (2)		I (2)	I (1), S (1)	R (1), S (1)	S (2)	S (2)	S (2)
11A	838 (3)	SLV Spain^{9V}-3	R (3)	I (1), S (2)	S (3)	S (3)	S (3)	S (3)
23A	42 (1)	DLV Tennessee^{23F}-4	I (1)	S (1)	S (1)	S (1)	S (1)	S (1)
	9752 (1)		S (1)	S (1)	R (1)	R (1)	R (1)	S (1)
6C	386 (2)	DLV Poland^{6B}-20	S (2)	S (2)	R (2)	R (2)	R (2)	S (2)
9V[b]	156 (1)	Spain^{9V}-3	I (1)	S (1)	S (1)	S (1)	S (1)	S (1)
13	9004 (1)		S (1)	S(1)	R (1)	S (1)	S (1)	S (1)

[a] Nonsusceptible isolates were defined as those displaying intermediate or complete resistance to at least one of the six antimicrobials tested for. PEN, penicillin; CTX, cefotaxime; TET, tetracycline; ERY, erythromycin; CLI, clindamycin; LVX, levofloxacin; R, resistant; I, intermediate; S, sensitive.
[b] PCV7/13 target serotypes.

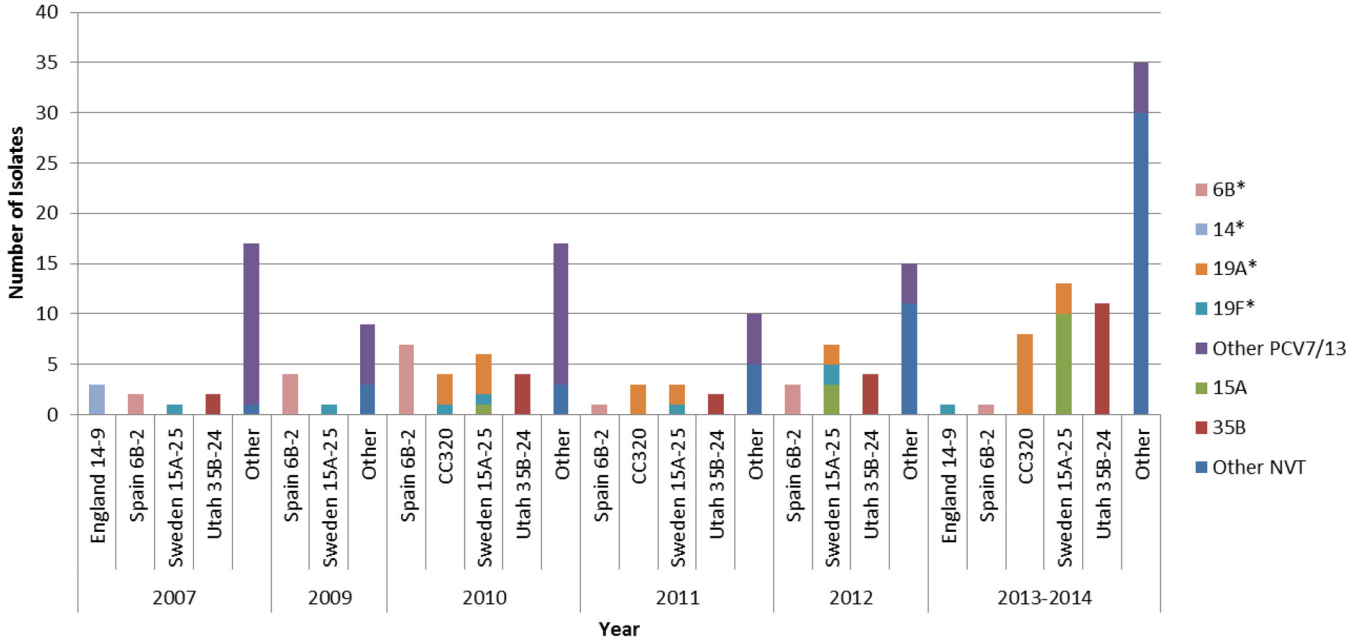

FIG 1 Changes in the most frequent noninvasive pneumococcal clones and their variants nonsusceptible to at least one antimicrobial in 2007 and 2009 to 2014. *, PCV7/13 serotype; PCV7, 7-valent pneumococcal conjugate vaccine serotypes; PCV13, 13-valent pneumococcal conjugate vaccine serotypes; NVT, non-vaccine-type pneumococci; CC, clonal complex. Common clonal complexes that expressed more than one serotype included Sweden^{15A}-25 (serotypes 15A, 19A, and 19F) and CC320 (19A and 19F). Other NVT pneumococci included nontypeable ($n = 15$), 33F ($n = 8$), 23B ($n = 7$), 15B/C ($n = 7$), 11A ($n = 3$), 23A ($n = 2$), 6C ($n = 2$), and 13 ($n = 1$).

their SLVs or DLVs were the most common sources of penicillin and erythromycin nonsusceptibility (Table 2). Spain^{6B}-2, its variants, and CC320 mostly expressed PCV7/13 serotypes 6B and 19A/19F, respectively. Both PCV13 serotype 19A and NVT 15A were associated with Sweden^{15A}-25 and its variants. Sequence type 558, an SLV of Utah^{35B}-24 expressing NVT 35B, was penicillin nonsusceptible but generally susceptible to other antimicrobials. However, two erythromycin-resistant ST558 isolates occurred in the latter period of the study. Sweden^{15A}-25, Spain^{6B}-2, and CC320 and their variants were the most common multidrug-resistant clones. Multidrug-resistant clone Sweden^{15A}-25 was mostly associated with NVT 15A. The remaining multidrug-resistant clones were mainly PCV7/13 serotypes. According to meningitis breakpoints, 45 isolates were cefotaxime nonsusceptible. If nonmeningeal breakpoints were applied, then just 6 isolates would be considered cefotaxime resistant.

Sweden^{15A}-25 isolates expressing serotype15A were among the most common source of nonsusceptibility in carriage (6/51) and nonbacteremic lower respiratory tract infection (6/46). Spain^{6B}-2 and its variants expressing serotype 6B were also associated with nonsusceptibility among the latter group (10/46; $P = 0.0020$) but declined in 2013 to 2014 (1/19). Serotype 19A of CC320 was a frequently carried nonsusceptible serotype (5/51) but was more associated with nonsusceptibility in otitis media (6/28; $P = 0.0142$). Serotype 35B of ST558 was a leading source of nonsusceptibility in carriage (8/51) and conjunctivitis (7/44). Nontypeable pneumococci of Norway^{NT}-42 and its variants were associated with nonsusceptibility in conjunctivitis (7/44; $P = 0.0034$) but infrequent in other sites ($n = 3$). Nonsusceptible Norway^{NT}-42 and its variants were isolated sporadically throughout the study and were not associated with outbreaks of conjunctivitis.

Changes in circulating nonsusceptible clones from 2007 versus 2009 to 2014. We compared antimicrobial-nonsusceptible clones and associated serotypes after PCV7 and PCV13 introduction to those collected in 2007 before PCV introduction (Fig. 1). Spain ^{9V}-3 and England ^{14}-9, the leading nonsusceptible clones in 2007, were infrequent following conjugate vaccine introduction (i.e., from 2009 to 2014). From 2009 to 2014, only three SLVs of Spain^{9V}-3 expressing serotype 11A and one Spain^{9V}-3 variant expressing serotype 9V occurred (Table 2). PMEN Spain^{6B}-2 and its variants significantly declined in 2014 compared to 2009 ($P = 0.0024$). CC320 did not occur in 2007 and 2009 but was frequent in 2010 and 2011. In 2012, CC320 was absent, but it did occur in 2013 to 2014 ($P = 0.0543$). Although not statistically significant, Sweden ^{15A}-25 and Utah ^{35B}-24 and their variants increased (Fig. 1). Interestingly, Sweden ^{15A}-25 was mostly associated with PCV7/13 serotypes 19F and 19A (13/16) in 2009 to 2012 but mostly expressed NVT 15A (7/10) in 2013 to 2014.

Genetic diversity among antimicrobial-nonsusceptible isolates. The Simpson's index of diversity and the Shannon index calculations revealed the pneumococcal STs/CCs associated with antimicrobial nonsusceptibility were quite diverse (Table 3). However, nonsusceptible STs/CCs were less diverse in 2007, before PCV7 introduction. The diversity of STs varied for common nonsusceptible serotypes. Serotype 35B was the least diverse, with a diversity (D) of 0.091 (confidence interval [CI], −0.079 to 0.260), while the diversities of nonsusceptible STs associated with 19A, 6B, and 15A were 0.679 (CI, 0.576 to

TABLE 3 Simpsons index of diversity (*D*) and Shannon's index (H′) of pneumococcal sequence types associated with noninvasive nonsusceptible pneumococcal isolates in Irish pediatric hospitals in 2007 and 2009 to 2014

Yr	Simpson's *D* (CI)	Shannon *H′*
2007	0.893 (0.794–0.993)	2.366
2009	0.978 (0.944–1.01)	2.441
2010	0.947 (0.919–0.975)	2.735
2011	0.965 (0.929–1.000)	2.552
2012	0.970 (0.945–0.996)	2.889
2013–2014	0.941 (0.913–0.969)	3.073

0.783), 0.786 (CI, 0.661 to 0.911), and 0.871 (CI, 0.783 to 0.959), respectively.

Prevalence of the pilus locus from 2009 to 2014. Of the 169 nonsusceptible pneumococci isolated from 2009 to 2014, 43% ($n = 74$) were positive for PI-1 and were associated with nonsusceptible PCV7/13 serotypes ($n = 46/82$) compared to nonsusceptible NVT serotypes ($n = 28/87$) ($P = 0.0020$). The most frequent PI-1-positive STs were ST558/SLV Utah^{35B}-24 ($n = 21$), ST320 ($n = 14$), ST94/SLV Spain^{6B}-2 ($n = 10$), and ST90/Spain^{6B}-20 ($n = 5$). Serotypes 35B (ST558/SLV Utah^{35B}-24), 11A (ST838/SLV Spain^{9V}-3), and 15B/C (ST7479) were the only nonsusceptible NVT pneumococci positive for PI-1.

DISCUSSION

We analyzed STs of nonsusceptible noninvasive pneumococci in Irish pediatric hospitals after PCV7 (2008) and PCV13 (2010) introduction. Important changes occurred in nonsusceptible pneumococcal clones from 2009 to 2014, compared with those from 2007, before PCV7 introduction. Notably, PMEN clones Spain^{9V}-3 and England^{14}-9 and their variants became infrequent or did not occur after conjugate vaccine introduction. The rapid decline of Spain^{9V}-3 and its variants in our population within a short period of PCV7/13 introduction differs from previous studies where Spain^{9V}-3 persisted (20, 21). The high vaccination coverage (92%) of PCV7/13 among Irish children may have contributed to a rapid decline in Spain^{9V}-3 compared to a lower coverage (30.7% to 55%) in regions where this clone persisted (20–22). Furthermore, we mostly included children born after universal conjugate vaccine introduction, whereas in the study by Gherardi et al. where Spain^{9V}-3 persisted, isolates were also obtained from adults likely not to be directly protected by conjugate vaccines (20).

A continuous decline was observed for Spain^{6B}-2, and encouragingly NVT serotypes were not associated with this clone. Conversely, three penicillin-nonsusceptible single-locus variants of Spain^{9V}-3 expressing NVT serotype 11A occurred in the latter period of our study. Expansion of clones expressing NVT may limit the anticipated benefit of conjugate vaccines in reducing nonsusceptible pneumococci.

As previously reported, nonsusceptible pneumococci within CC320 were absent among noninvasive pneumococci before PCV7 introduction (23, 24). However, in 2010 to 2011, nonsusceptible CC320 pneumococci expressing serotype 19A increased in circulation, as reported elsewhere after PCV7 introduction (23, 25).

It has been suggested that pneumococcal clonal diversity may lead to the successful spread of resistance mechanisms (26). In our study, STs among nonsusceptible pneumococci were diverse. However, there was less diversity of antimicrobial-nonsusceptible STs before conjugate vaccine introduction than after conjugate vaccine introduction, suggesting that potential emerging nonsusceptible STs/clones may not yet be fully established in our population. Interestingly, there were low levels of diversity among STs associated with serotype 35B, as described elsewhere (27). Similar to what was reported in Spanish and American studies, penicillin-nonsusceptible serotype 35B of ST558 was common in our study and increased following PCV7 and PCV13 introduction (27–29). In 2013 to 2014, two erythromycin- and penicillin-nonsusceptible 35B/ST558 isolates occurred in our study. If continued clonal expansion of these coresistant pneumococci occurs, it may present a potential problem for empirical treatment of noninvasive infection.

Among our multidrug-resistant isolates, Sweden^{15A}-25 and its variants increased following PCV introduction. Similar to ST558, Sweden^{15A}-25 and its clonal variants have undergone successful clonal expansion (27). In the initial years following PCV7 introduction, this clone was associated mostly with PCV13 serotype 19A. However, by the end of the study Sweden^{15A}-25 and its variants mostly expressed NVT serotype 15A. The occurrence of Sweden^{15A}-25 expressing different capsular serotypes is well documented (21, 30). Importantly, NVT expressed by Sweden^{15A}-25 variants, including those not traditionally associated with antimicrobial nonsusceptibility, could lead to antimicrobial nonsusceptibility rates similar to those of the pre-PCV7 era (21, 30).

The PI-1 locus has been associated with the spread of antimicrobial-nonsusceptible clones, including Spain^{9V}-3 and England^{14}-9 and CC320 (5, 21, 31). Moreover, nonsusceptible piliated clones have spread in countries with low antibiotic use (5). In our study, PI-1 was mostly associated with clones expressing PCV7/13 serotypes. The low frequency of PI-1 in nonsusceptible NVT may help limit the spread of these clones.

There were some limitations to our study. Vaccination and antibiotic consumption history for patients was unavailable, and data from 2009 to 2012 was limited to one hospital. Furthermore, pneumococcal strains from carriage and noninvasive infection from children presenting to hospitals may select for more resistant clones, rather than clones more representative of healthy children in the community. Nonetheless, our study illustrates important findings. Introduction of conjugate vaccines to the childhood immunization schedule has resulted in the reduction of clones Spain^{9V}-3, England^{14}-9, and Spain^{6B}-2 associated with PCV7/13 serotypes 14, 9V, and 6B, respectively. This suggests that conjugate vaccines are positively impacting these clones. The increase of Sweden^{15A}-25 and Utah^{35B}-24 and their variants expressing NVT serotypes may lead to continued high antimicrobial resistance rates among noninvasive pneumococci. It is concerning that these nonsusceptible clones associated with NVT pneumococci have occurred in several countries after conjugate vaccine introduction (32, 33). However, the absence of the PI-1 in some nonsusceptible NVT clones is encouraging as this may limit their dissemination. Overall, continued surveillance will determine if nonsusceptible clones, such as Sweden^{15A}-25 and Utah ^{35B}-24 and their variants, or others expressing NVT, increase in pediatric noninvasive infection, with possible implications for dissemination to the adult population and potential issues in effective antimicrobial treatment.

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M.M.E. performed experiments, analyzed the data, and drafted the manuscript. H.H. was involved in the design of the study, its supervision, and the drafting of the manuscript. R.C. was involved in the design and supervision of the study. M.C. was involved in the design of the study. I.V. was involved in study concept and design and was a technical supervisor for the project. M.M. was a technical supervisor for the project and was involved in drafting the manuscript. All authors have read and approved the final manuscript.

As potential conflicts of interest, H.H. has recent research collaborations with Pfizer and has also recently received lecture and other fees from Novartis, Pfizer, AstraZeneca, and Astellas, and I.V. has received funding support from Pfizer.

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