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ORIGINAL ARTICLE

Serotype 19A and 6C Account for One-Third of Pneumococcal Carriage Among Belgian Day-Care Children Four Years After a Shift to a Lower-Valent PCV

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Background: Pneumococcal conjugate vaccines (PCVs) effectively reduce infection and asymptomatic carriage of *Streptococcus pneumoniae* vaccine serotypes. In 2016, Belgium replaced its infant PCV13 program by a 4-year period of PCV10. Concomitantly, *S. pneumoniae* serotype carriage was monitored together with the carriage of other nasopharyngeal pathogens in children attending day-care centers.

Methods: From 2016 to 2019, a total of 3459 nasopharyngeal swabs were obtained from children aged 6–30 months. Culture and qPCR were used for the identification of *S. pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* and for serotyping and antimicrobial susceptibility assessment of *S. pneumoniae* strains.

Results: *S. pneumoniae* colonization was frequent and stable over the study years. *H. influenzae* and *M. catarrhalis* were more frequently carried ($P < .001$) than *S. pneumoniae*, by, respectively, 92.3% and 91.0% of children. Prevalence of all PCV13 serotypes together increased significantly over time from 5.8% to 19.6% ($P < .001$) and was attributable to the increasing prevalence of serotype 19A. Coincidently, non-vaccine serotype 6C increased ($P < .001$) and the overall pneumococcal non-susceptibility to tetracycline and erythromycin. Non-susceptibility to cotrimoxazole decreased ($P < .001$).

Conclusions: The switch to a PCV program no longer covering serotypes 19A, 6A, and 3 was associated with a sustained increase of serotypes 19A and 6C in healthy children, similarly as in invasive pneumococcal disease. This resulted in a re-introduction of the 13-valent conjugate vaccine during the summer of 2019.

Key words: children; day-care center; PCV10; PCV13; pneumococcal carriage; serotypes.

Asymptomatic nasopharyngeal carriage of potentially pathogenic bacteria represents the primary reservoir of bacterial species within a population and is considered a precursor for the development of major childhood diseases [1]. *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are the predominant causes of otitis media [1]. *S. pneumoniae* is a major cause of morbidity and mortality worldwide, especially in young children and the elderly, mainly by respiratory infections such as acute otitis media (AOM) and pneumonia, but also invasive diseases such as bacteremia and meningitis.

The introduction of pneumococcal conjugate vaccines (PCVs) in infant vaccine programs was very effective in reducing disease caused by the serotypes included in the vaccines. Besides inducing direct protection in recipients, PCV vaccine programs also reduce the carriage and transmission of vaccine serotypes resulting in herd protection [2]. In addition, PCVs can provide cross-protection against some vaccine-related serotypes, particularly to serotypes belonging to the same serogroups [3]. However, overall pneumococcal carriage prevalence is minimally affected due to serotype replacement by non-vaccine pneumococcal serotypes [1]. This change in the composition of the pneumococcal reservoir may affect interactions with other common respiratory pathogens [4].

A universal PCV10 vaccination schedule is utilized in European countries such as the Netherlands and Finland, which has resulted in high protection against carriage of PCV10 serotypes with limited serotype replacement over time, and protection against invasive pneumococcal disease (IPD) caused by PCV10 serotypes [3, 5]. The history of PCV use in infants

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Table 1. Demographic and Clinical Characteristics of Children Sampled in Belgian Day-Care Centers (DCC)

		DCC			
		Year 1 March 2016–July 2016 *n* = 760	Year 2 Nov 2016–March 2017 *n* = 902	Year 3 Nov 2017–March 2018 *n* = 953	Year 4 Nov 2018–March 2019 *n* = 844
Region	Flanders	332; 43.7%	488; 45.2%	552; 58.0%	458; 54.2%
	Wallonia	353; 46.4%	287; 31.8%	282; 29.6%	273; 32.3%
	Brussels	75; 9.9%	127; 14.1%	119; 12.5%	113; 13.4%
Age (months)	6–12	98; 12.9%	217; 24.1%	209; 21.9%	198; 23.5%
	13–24	415; 54.6%	457; 50.7%	528; 55.4%	427; 50.6%
	25–30	247; 32.5%	228; 25.2%	216; 22.7%	217; 25.7%
Sex	Male	387; 50.9%	455; 50.4%	469; 49.2%	434; 51.4%
Vaccination status	Fully PCV13	582; 76.6%	354; 39.2%	30; 3.1%	3; 0.4%
	Fully PCV10	0; 0.0%	107; 11.9%	725; 76.1%	732; 86.7%
	Mix	29; 3.8%	133; 14.7%	117; 12.3%	8; 0.9%
	Incomplete/Unknown/ not vaccinated	149; 19.6%	308; 34.1%	81.0; 8.5%	101; 12.0%
AOM-history	Yes	258; 33.9%	225; 24.9%	199; 20.9%	178; 21.1%
AB < 3 months	Yes	248; 32.6%	254; 28.2%	217; 22.8%	221; 26.2%
Common cold symptoms	Yes	169; 22.2%	344; 38.1%	429; 45.0%	508; 60.2%

Number and proportion of children per period and per variable are shown. *n* = number; PCV = pneumococcal conjugate vaccine; DCC = day-care center; AB = antibiotics; AB < 3 months: antibiotic use in the 3 months prior to sampling. Mix = age appropriately vaccinated, schedule started with PCV13 and completed with PCV10.

in Belgium has created an interesting context to study pneumococcal serotype replacement and nasopharyngeal colonization dynamics, complementary to invasive disease surveillance. Pediatric pneumococcal vaccination was introduced in 2004 and was implemented in the nation's childhood vaccination program in a 2 + 1 schedule with PCV7 in 2007. In 2011, PCV7 was replaced by PCV13, which was in turn replaced by PCV10 after 4 years and from the summer of 2019, PCV13 was re-introduced in the vaccination program. NP swabs are the preferred samples to investigate pneumococcal carriage since the nasopharynx is the primary reservoir for *S. pneumoniae* in children [6], and they allow for sensitive detection of other colonizers using molecular methods [4, 6].

In the present study, NP samples were collected yearly following the switch in the vaccination programs from PCV13-to-PCV10. All data mentioned in the present manuscript are obtained during the PCV10 period. The aim of the study was to assess in detail consecutive changes in prevalence and in the antibiotic (AB) susceptibility profile of all and vaccine-specific *S. pneumoniae* serotypes (PCR-based analysis) in the nasopharynx of healthy children attending day-care centers (DCCs) over a 4-year period since 2016. In order to investigate co-carriage, colonization patterns of *Staphylococcus aureus*, *H. influenzae*, and *M. catarrhalis* were also monitored.

METHODS

Ethical Statement

The study was approved by the ethics committee of Antwerp University (UA) and the Antwerp University Hospital (UZA, ID 15/45/471 and ID 18/31/355). Written informed consent and a completed questionnaire including demographics, clinical characteristics, and vaccination status were obtained from the infants' parents or legal representatives at the time of initial enrolment.

Study Population

Children were recruited in DCCs randomly selected over the three Belgian regions (Table 1). Healthy children between 6 and 30 months old that were not treated with oral ABs in the seven days before sampling were included (see [7] for detailed inclusion and exclusion criteria). Children are defined as healthy if they are healthy enough to be present in a DCC. These are children who either do not have any condition or do have a condition that is fully controlled which allows the child to come to the DCC. In the first period, an additional age criterium was applied in Flanders and Brussels so as to include exclusively children who received PCV13 as their primary vaccine dose. As a result, children were on average older in the first period compared to children recruited in subsequent periods [8]. Children were sampled according to WHO recommendations [6].

Sampling and Sample Processing

A single NP swab was taken with a flocked nylon swab, put in 1 ml STGG (Skim milk—Tryptone—Glucose—Glycerol), and cultured or stored at -80°C [8] to be processed later by both culture and PCR.

Culture Analysis

Samples were plated on blood agar to detect *S. pneumoniae* with or without enrichment in brain-heart infusion. *M. catarrhalis* (up to the 3rd year) and *S. aureus* were detected on the same plates, and

Table 2. Carriage of *S. pneumoniae, H. influenzae* and *M. catarrhalis* in Children Attending Day-Care Center (DCC) (2016–2019) in Belgium

	DCC *n* = 3459
S. pneumoniae	2713; 78.4%
H. influenzae	3191; 92.3%
M. catarrhalis	3148; 91.0%
S. aureus	146; 4.2%
S. pneumoniae—H. influenzae—M. catarrhalis	2392; 69.2%
H. influenzae—M. catarrhalis	2921; 84.4%
S. pneumoniae—H. influenzae	2529; 73.1%
S. pneumoniae—M. catarrhalis	2556; 73.9%

Numbers and proportions of children attending day-care centers and positive for at least one of the three pathogens studied are shown. Carriage is calculated as a combination of culture and PCR results. *n* = number; AOM = acute otitis media; DCC = day-care centers.

samples were plated in parallel on homemade horse blood agar including bacitracin, vancomycin, and factor V supplement to identify *H. influenzae*. Pneumococcal strains were serotyped using the Quellung reaction with serotype-specific sera (SSI Diagnostica, Hillerod, Denmark). Antimicrobial susceptibility for erythromycin, penicillin, tetracycline, and cotrimoxazole was tested by disk diffusion. If disc diffusion showed non-susceptibility for penicillin, the minimum inhibitory concentration (MIC) was determined by Etest (Biomérieux, Craponne, France). A MIC of > 0.06 mg/L for penicillin was interpreted as non-susceptible [8].

Molecular Detection of *H. influenzae* and *M. catarrhalis* and Quantification of *S. pneumoniae*

DNA was extracted using the automated NucliSENS® EasyMag® (Biomérieux), following proteinase K pre-treatment [8]. Up to the third year, the density of pneumococcal DNA was determined using quantitative Taqman real-time PCR (qRT-PCR) targeting *lytA* [8, 9]. The presence of *H. influenzae* and *M. catarrhalis* DNA was detected by real-time PCR targeting the genes *P6* [10] and *copB* [1], respectively, in samples culture-negative for *H. influenzae* and *M. catarrhalis* until year 3. In year 4, the presence of *H. influenzae* and *M. catarrhalis* was detected by real-time PCR in all collected samples. Samples were classified as positive when C_T values were ≤ 35.

Molecular In-Sample Serotyping of *S. pneumoniae*

All *lytA*-positive samples were subjected to molecular serotyping by real-time PCR using previously published primers and probes for all serotypes included in PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F) [11–13]. For serogroup 6, the genes *wciP* and *wciN* were analyzed by three real-time PCRs targeting, respectively, the single nucleotide polymorphism at codon 195 [11, 12] and the *wciN* gene [13]. Samples were pooled and screened for the presence of pneumococcal vaccine serotypes. If found positive, pooled samples were unpooled and the individual sample was determined. Serotype-specific PCRs were classified as positive when C_T values were ≤ 35.

Samples were considered *S. pneumoniae*-positive if *S. pneumoniae* was detected either in culture or with molecular analysis. When interpreting the results, it is important to keep in mind that the used microbiological protocols can differ depending on what has to be determined. Serotypes were determined by performing culture-based and/or real-time PCR-based analysis, while AB non-susceptibility was only performed on pneumococci obtained in culture.

Statistical Analysis

The sample size was calculated using the R-package "power" [8] to achieve 80% power to detect a 4% difference in carriage prevalence of three pneumococcal vaccine serotypes (19A, 6A, and 3) over four years (2016–2019) (with type I error, α = .05) [8]. The chi-square (χ^2) test was used to assess significant differences in carriage prevalence (α = .05). To identify carriage predictors of *S. pneumoniae*, serotype 19A, and 6C and to investigate time trends of non-susceptibility to ABs, multiple binary regressions were performed (adjusted by generalized estimating equations (GEEs)). The GEE model analyses were performed using the R-package "geepack" [14]. Only variables that were significant in univariate analyses were included in the multiple regression analysis. Missing values were not replaced.

RESULTS

Over the four-year study period (2016–2019), 3459 NP samples obtained from healthy infants attending DCCs were analyzed by conventional culture (*n* = 3273) and/or real-time PCR (*n* = 3459).

High (Co)-Carriage Rates of *S. pneumoniae, M. catarrhalis,* and *H. influenzae* in DCC-Children

Carriage of *S. pneumoniae* remained stable and high during the study period. *H. influenzae* and *M. catarrhalis* were carried most frequently (Table 2 and more detailed in Supplementary Table 4), whereas *S. aureus* was uncommon (Table 2). Yearly fluctuations were non-significant for all pathogens, except *H. influenzae* for which carriage was lower during the first study period compared to the other periods (Figure 1) ($P < .001$). All but 22 samples were positive for at least one pathogen (99.4%), and co-colonization of the three most frequent pathogens was very common (69.2%). Co-carriage of *M. catarrhalis* with *H. influenzae* (84.4%) was most frequent and they both co-occurred with *S. pneumoniae* at similar rates (Table 2). Conversely, colonization by only one of the investigated pathogens was rather rare (Figure 1).

Increase in PCV13 Vaccine Serotypes in DCC-Children

Carriage of PCV13 serotypes among *S. pneumoniae*-positive DCC samples (n = 2713) increased significantly from 2016 to 2018–2019 ($P < .001$) (Figure 2), especially that of serotype 19A of which prevalence almost doubled from the third to the

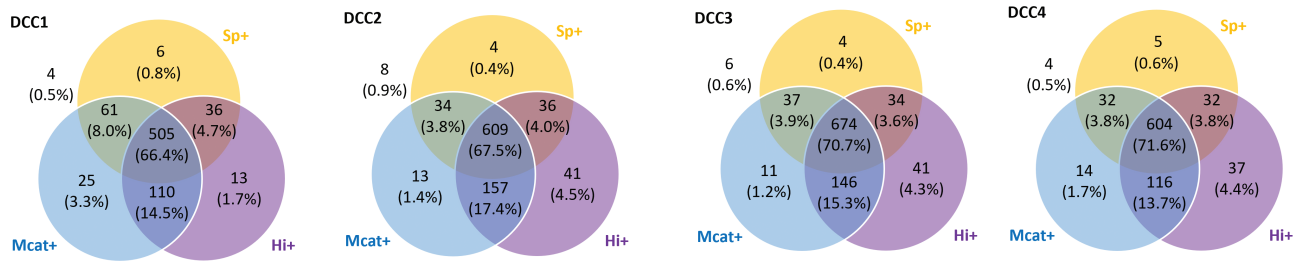

Figure 1. Co-carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, by study year in children attending DCC. Numbers and proportions of children attending day-care and positive by culture or PCR for at least one of the three pathogens studied are shown. Four study years are shown (DCC1–DCC4). DCC=day-care centre; Mcat+ = positive for *M. catarrhalis* (blue); Hi+ = positive for *H. influenzae* (purple); Sp+ = positive for *S. pneumoniae* (yellow).

fourth study year ($P < .001$). Serotype 3 showed low carriage rates, however, a non-significant increase from 0.7% in 2016 to 1.3% in 2018–2019 could be observed. Conversely, the prevalence of PCV10 serotypes decreased non-significantly among *lytA*-positive samples, with serotype 19F being the predominant PCV10 serotype (Figure 2).

Interestingly, also the carriage prevalence of serotype 6C, which is closely related to the PCV13-non-PCV10 serotype 6A, increased significantly from 1.5% (9/608) in 2016 to 15.9% (107/673) in 2018–2019 ($P < .001$). Detailed information on the distribution of all PCV13 vaccine serotypes can be found in Supplementary Table 1.

Increasing Non-Susceptibility to Tetracycline and Erythromycin in Pneumococcal Strains Carried by DCC-Children

Non-susceptibility to tetracycline and erythromycin increased ($P < .001$) over the study years; whereas that of cotrimoxazole decreased ($P < .001$) (Table 3). No changes were found in non-susceptibility to penicillin from year 2 onward (Table 3). Importantly, pneumococcal non-susceptibility to more than one AB increased significantly (year 1: 12.3%; year 2: 11.2%; year 3: 17.7%; year 4: 32.8%) ($P < .001$).

Predictors of *S. pneumoniae*, Serotype 19A and Serotype 6C Carriage Using Multiple Regression Analyses

Carriage of *S. pneumoniae* was positively associated with the child's sex (with girls carrying *S. pneumoniae* more often than boys), having siblings, or having common cold symptoms, and negatively associated with having received ABs in the past three months. *H. influenzae* or *M. catarrhalis* colonization also appeared to positively impact *S. pneumoniae* carriage; while the opposite was true for *S. aureus* (Supplementary Table 2).

Also, characteristics associated with serotypes 19A and 6C carriage were investigated in separate multiple regression

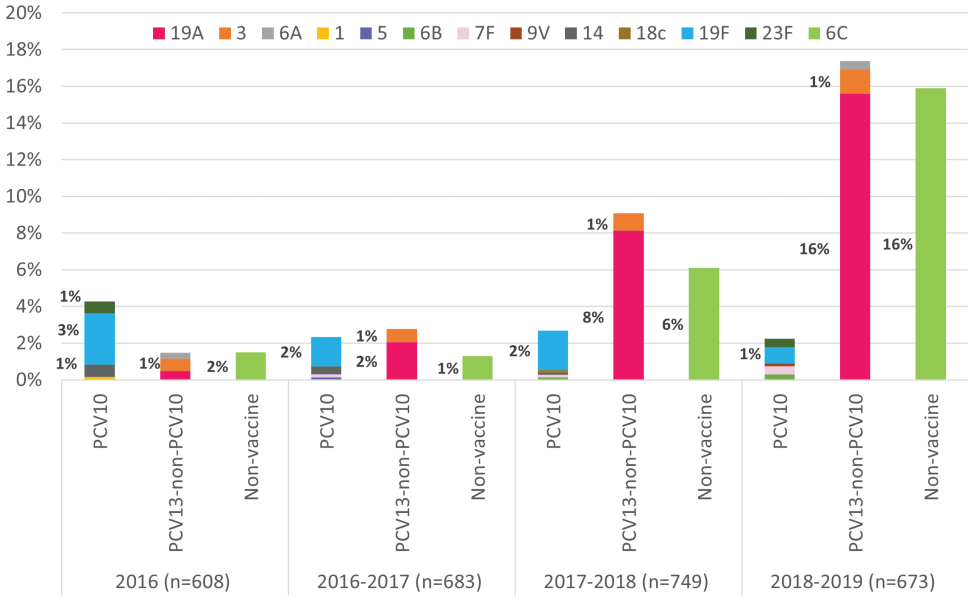

Figure 2. Carriage prevalence of PCV13 serotypes and 6C in *lytA*-positive samples from children attending DCC. Prevalence of PCV13 serotypes (1, 5, 3, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) and non-vaccine serotype 6C in *lytA*-positive children during the period 2016–2019 by either culture or real-time PCR combined. Exact numbers and percentages for all serotypes can be found in Supplementary Table 4.

Table 3. Antimicrobial Non-Susceptibility to Penicillin, Tetracycline, Erythromycin, and Cotrimoxazole in Pneumococcal Culture-Positive Samples

	Year 1 2016 *n* = 463	Year 2 2016–2017 *n* = 752	Year 3 2017–2018 *n* = 668	Year 4 2018–2019 *n* = 469	*P*-value (chi²)
Penicillin	62; 13.4%	149; 19.8%	123; 18.4%	91; 19.4%	.03
Tetracycline	54; 11.7%	86; 11.4%	133; 19.9% %	121; 25.8%	**<.001**
Erythromycin	80; 17.3%	121; 16.1%	150; 22.5%	122; 26.0%	**<.001**
Cotrimoxazole	163; 35.2%	298; 39.6%	206; 30.8%	97; 20.7%	**<.001**

Numbers and percentages of pneumococcal strains that are non-susceptible to penicillin, tetracycline, erythromycin, and cotrimoxazole for the four study years. In year 4, not all samples were culture analyzed due to feasibility reasons, but a random selection of 700 samples. *n* = number of strains analyzed by culture.

models, as they were among the most common serotypes in the fourth study year (Supplementary Table 3). These models confirmed the significant increase of both serotypes over time. Serotype 19A was more frequently carried in Flanders (the northern part of Belgium) than in the Walloon region (the southern part of Belgium). Serotypes 19A and 6C were both less frequently non-susceptible to cotrimoxazole than other carried serotypes; moreover, 6C was more frequently non-susceptible to tetracycline and erythromycin.

DISCUSSION

The present study shows a clear increase of PCV13-non-PCV10 serotypes in a nasopharyngeal carriage in healthy children attending DCCs, together with an overall low prevalence of PCV10 serotypes. The prevalence of both the PCV13-non-PCV10 vaccine serotype 19A and the non-vaccine serotype 6C increased with time. The increase in serotype 6C appeared to be associated with an increasing non-susceptibility to tetracycline and erythromycin with time, which was not the case for 19A.

Previously, our group already reported an increase in PCV13-non-PCV10 vaccine serotypes in children attending DCC and children suffering from AOM from 2016 to 2018, which was also largely attributable to an increasing prevalence of serotype 19A [15, 16]. Since then, monitoring of pneumococcal carriage was continued in Belgian children attending DCCs. This revealed a sustained increase in serotype 19A as is shown in the present study and is likely due to the switch in the Belgian PCV program (PCV13 to PCV10) in 2016. A Brazilian NP-carriage study investigating the long-term impact of PCV10 on pneumococcal colonization observed this increase only seven years after the introduction of PCV10 [17]. This slower increase might be related to a difference in baseline prevalence of serotype 19A: PCV10 was the first pneumococcal vaccine implemented in Brazil, while Belgium had a history of PCV7 prior to PCV13. The significant increase of serotype 19A after the switch to PCV10 was also observed in children with invasive pneumococcal disease resulting in a rise in pediatric IPD cases from 2017 onwards [18]. In 2020, serotype 19A accounted for 19.3% of the IPD cases in Belgium, which was an increase of 3.8% from 2019 [19]. As a result of the changing dynamics of IPD-associated pneumococcal serotypes, the Belgian Superior Health Council revised its PCV recommendation in 2019 and advised to again use PCV13 in the Belgian childhood vaccination program [20].

Simultaneously, also a steep increase in the prevalence of serotype 6C carriage was observed. Even though serotype 6C is not part of PCV13 it is closely related to the PCV13-vaccine serotype 6A and cross-protection between the two serotypes has been proposed [21, 22]. An increase in the carriage prevalence of serotype 6C was also reported following the introduction of PCV10 in Iceland and Brazil [17, 23]. Importantly, in 2020 serotype 6C was responsible for 5.9% of reported IPD cases in Belgium [19], and constituted one of the predominant IPD-causing serotypes. Consecutive to its increasing carriage prevalence, an increase in IPD cases caused by serotype 6C was observed from 2019 to 2020 [19]. In the present study, serotype 6C appeared to be more often non-susceptible to tetracycline and erythromycin compared to other serotypes, a finding also shown by the emergence of multi-drug resistant carriage isolates in Brazil [24]. It is known that macrolide resistance genes (*erm*B) and tetracycline resistance genes (*tet*M) are very often found on the same mobile element and can lead to co-selection [25]. However, in Belgium there is a decrease in macrolide consumption from 2015 onward thus this finding does not explain the increasing non-susceptibility to tetracycline [26]. Similar findings are observed in IPD-causing isolates in Belgium in 2019, where 64% of serotype 6C showed a reduced erythromycin susceptibility, and 6% showed a reduced penicillin susceptibility which was higher than in carriage strains (1.7%) [19].

Our study, as well as others, does not support the potential cross-protection of PCV10 vaccine types 6B and 19F against 6C and 19A, respectively, for carriage [27]. Previous studies have indeed described a decreasing prevalence of IPD cases caused by 19A following the introduction of PCV10, suggesting some level of cross-protection against 19A [28]. However, continued long-term surveillance revealed an increasing prevalence of 19A-associated IPD cases [29]. Besides, higher levels of cross-protective antibodies might be needed to protect against carriage than for IPD [21]. Cross-protection against 6C carriage by the 6A antigen in PCV13 is confirmed by our data as 6C prevalence was low at the start and increased after the

introduction of PCV10. This finding is further supported by a study investigating the IPD incidence after the introduction of the different PCVs in the childhood immunization program in Sweden, showing that 6A, and not 6B, confers cross-protection to 6C [30].

Overall *S. pneumoniae* and *M. catarrhalis* carriage remained stable over all study years and changes in *H. influenzae* carriage were minor, in contrast with the clear dynamics of *S. pneumoniae* serotypes. This observation indicates that serotype replacement occurs and is further stimulated in the present population by the high frequency and close contact among children in DCCs [28]. Subsequent co-colonization analyses highlighted positive associations between *S. pneumoniae* and *M. catarrhalis* or *H. influenzae*, which is in line with previous reports [4]. In contrast to *M. catarrhalis* and *H. influenzae*, a negative association between *S. pneumoniae* and *S. aureus* has been reported [4], as in the present study.

The positive associations between *S. pneumoniae* and *H. influenzae* or *M. catarrhalis* might be established by the passive protection of other pathogens against AB killing [31], or by differences in host susceptibility and ethnicity [1]. For example, *M. catarrhalis* has been reported to confer β-lactamase protection to *S. pneumoniae* while, conversely, *S. pneumoniae* provides passive protection to *M. catarrhalis* from macrolide killing [31, 32]. Children carrying *S. pneumoniae* and *H. influenzae* together can have a reduced serotype diversity compared to children carrying only *S. pneumoniae* as co-colonization with *H. influenzae* increases with the immunologic evasiveness and the metabolic efficiency of pneumococcal serotypes [33]. Piliated *S. pneumoniae* strains may either induce a host immune response that is harmful to *S. aureus* colonization, or just adhere better and thus inhibit *S. aureus* colonization [34], although other inhibitory mechanisms have been postulated as well [35].

A close follow-up of pneumococcal colonization, serotype prevalence, and co-carriage of other respiratory pathogens is especially important in Belgium, where PCV implementation has changed from PCV13 to PCV10 in 2015–2016, and back to PCV13 in 2019. The increasing trend in the prevalence of PCV13 vaccine serotypes observed in the present study stresses the importance of continuous surveillance. As the nasopharynx is a polymicrobial environment the interactions that prevent or promote co-colonization might be important in the pathogenesis of respiratory infections [36]. Thus, monitoring co-colonization profiles in children could provide more insights into the interactions at play and the impact of the pneumococcal vaccination program.

In conclusion, we observed a high and stable carriage of *S. pneumoniae* coupled with increasing proportions of PCV13-non-PCV10 serotypes after the PCV13 to PCV10 vaccine switch during 2016–2019 in DCC-children, and predominated by serotype 19A, followed by serotype 6C. The consistent trend and the clear association in time indicate the vaccine switch as the cause of these changes. For that reason, the Belgian government decided to reintroduce PCV13 from the summer of 2019 onward. Continued surveillance will demonstrate whether the use of PCV13 results in a decrease in these serotypes.

Supplementary Data

Supplementary materials are available at the *Journal of The Pediatric Infectious Diseases Society* online (http://jpids.oxfordjournals.org).

Notes

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