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RESEARCH ARTICLE

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Back to the future? Drastic drop in serotype 19A carriage in daycare centers within two years after a second switch to PCV13 in Belgium

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ABSTRACT

Pneumococcal conjugate vaccines (PCVs) reduce *Streptococcus pneumoniae* infection and carriage. After switching from PCV13 to PCV10 in 2015–2016, Belgium switched back to PCV13 in 2019. Building on our systematic monitoring of childhood nasopharyngeal carriage since 2016, here, we analyze the serotypes of *S. pneumoniae* and other pathogens in children attending daycare centers (DCCs) from 2018 to 2021. From the period of 2018–2019 to 2020–2021, we included a total of 2,741 nasopharyngeal swabs collected from children aged 6 to 30 months. We identified *S. pneumoniae*, *Haemophilus influenzae, Moraxella catarrhalis*, and *Staphylococcus aureus* and conducted serotyping and antimicrobial susceptibility assessments of *S. pneumoniae* strains using culture methods and real-time PCR. *S. pneumoniae* carriage was frequent and quite stable over the three study years. *H. influenzae* and *M. catarrhalis* were more frequently carried than *S. pneumoniae*. Frequency of all PCV13-serotypes together among *S. pneumoniae* carriers decreased significantly from 19.4% in 2018–2019 to 9.9% in 2020–2021 ($p < .001$), largely due to the decreased serotype 19A carriage. Resistance of pneumococcal strains to penicillin increased significantly over the three study years. Two years after the second switch to PCV13 in 2019, pneumococcal serotype 19A carriage decreased again significantly in Belgian children attending daycare centers.

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Introduction

Streptococcus pneumoniae is an important pathogen causing high morbidity and mortality in young children mainly by respiratory infections such as acute otitis media (AOM) or pneumonia but also by invasive pneumococcal disease (IPD).[1,2] Asymptomatic carriage of potentially pathogenic bacteria such as *S. pneumoniae* is the primary reservoir of bacterial species within a population, and as such, considered a precursor for the development of these childhood diseases.[1,2]

Although there are currently more than 100 pneumococcal serotypes identified, some specific serotypes are more frequently involved in the development of IPD.[3] After the introduction of pneumococcal conjugate vaccines (PCV) in infant vaccination programs a very effective reduction was seen in pneumococcal disease caused by the serotypes included in the vaccines.[4] PCV vaccination programs were not only important in conferring direct protection in recipients but also in reducing carriage and transmission of vaccine serotypes resulting in herd protection.[3–5] Overall pneumococcal carriage prevalence was minimally affected due to serotype replacement by non-vaccine pneumococcal serotypes.[1] This change in composition of the pneumococcal reservoir may, however, affect interactions with other common respiratory pathogens such as respiratory syncytial virus (RSV).[5,6]

In Belgium, the pediatric PCV program has created an interesting context to study pneumococcal serotype replacement and nasopharyngeal colonization dynamics, in complement with invasive disease surveillance. In 2004, the pediatric pneumococcal vaccination was introduced and in 2007 PCV7 was implemented in each region's childhood vaccination program according to a 2 + 1 schedule. Two primary doses are given at the age of 8 and 16 weeks, the booster dose at the age of 12 months. For preterm infants (<37 weeks) and infants with known immunodeficiency, a 4-dose schedule (3 + 1) with an additional dose at 12 weeks of age is recommended.[5,7] PCV7 was replaced by PCV13 in 2011, which was in turn replaced by PCV10 in 2016. From summer 2019, PCV13 was re-introduced in the vaccination program due to a significant increase of 19A IPD. This unique sequence highlights the importance of systematically monitoring nasopharyngeal pneumococcal carriage in healthy infants next to IPD, to respond to resurgence of pneumococcal strains after a vaccine change, and to inform policy makers who consider changing a pneumococcal vaccine.

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In the present study, nasopharyngeal samples were collected yearly following the switches in the pediatric vaccination program in Belgium. Nasopharyngeal swabs are used to investigate pneumococcal carriage since: (1) the nasopharynx is the primary reservoir for *S. pneumoniae* in children[5,7] and (2) sensitive detection of other colonizers, such as *Moraxella catarrhalis* and *Haemophilus influenzae*, are possible with molecular testing.[6,8] During the PCV10 period (2016–2019), we observed a significant increase in the PCV13 vaccine serotype 19A and the vaccine-related serotype 6C.[5] In this study, data from the last year of the PCV10 period as well as data collected during the first two years of the second PCV13 period were used. The main aim of the study was to investigate if the second switch to PCV13, after four years of PCV10 period, contributed to changes in pneumococcal prevalence with a focus on PCV13 vaccine serotypes in the nasopharynx of healthy children attending daycare centers (DCCs). The secondary aims were to investigate the antibiotic (AB) susceptibility profile of all carried pneumococci serotypes as well as vaccine-specific serotypes over a three-year period (2018–-2019, 2019–2020, 2020–2021) and to monitor the colonization patterns of *Staphylococcus aureus*, *H. influenzae*, and *M. catarrhalis* to investigate co-carriage patterns. Since the COVID-19 pandemic affected social contact behavior from March 2020 onwards,[9,10] we also tried to disentangle the potential impact of SARS-COV-2 in children in terms of pneumococcal carriage, antimicrobial susceptibility, and co-carriage patterns.

Methods

Ethical statement

Ethical approval from the ethics committee of University of Antwerp (UA) and the Antwerp University Hospital (UZA, ID 15/45/471 and ID 18/31/355) was obtained. Written informed consent and a completed questionnaire from the parents or legal representatives at the time of initial enrollment were also received.

Study population

Details about sample size determination and daycare center recruitment are given in Wouters et al.[11] and summarized here. Healthy children aged between 6 and 30 months were recruited in daycare centers (DCCs) which were randomly selected in each of the three Belgian regions, with the number of centers proportionate to the regional population. There were no selection criteria for the DCCs. The primary objective of the study was to detect a change in the proportion of PCV13-non-PCV10 serotypes (19A, 6A, and 3) in *S. pneumoniae* carriers by PCR. To achieve this, a minimum of 707 children were to be recruited per year. Children that were not treated with oral antibiotics (AB) in the seven days before sampling were included in the present study (for detailed inclusion and exclusion criteria see Wouters et al.[11]). Children were defined healthy if they were healthy enough to attend a daycare center. This included children without a debilitating condition or with a fully controlled condition allowing the child to attend the daycare center. Every season, new children were recruited, implying this was not a longitudinal cohort study. We also use strategies to detect and minimize selection bias, for example, comparison of demographic characteristics with previous study periods and the inclusion of many daycare centers that are geographically spread over the regions.

Sampling and sample processing

A trained study nurse collected a questionnaire that included clinical and demographic characteristics of the study participants. The vaccination status of the child was based on vaccination records (baby clinic booklet) or Vaccinet. A nasopharyngeal swab was taken with a flocked nylon swab, according to WHO-recommendations.[7,8] The swab was put in 1 ml STGG (Skim milk – Tryptone – Glucose – Glycerol), and cultured or stored at −80°C[7] to be processed later by both culture and PCR. Details about sample transport and storage are previously described by Wouters et al.[11]

Culture analysis

Samples were plated on blood agar (with or without enrichment in brain-heart infusion (BHI)) to detect *S. pneumoniae* and *S. aureus*. *S. pneumoniae* strains were serotyped using the Quellung reaction with serotype-specific sera (SSI Diagnostica, Hillerod, Denmark). For reasons of feasibility, a random selection of 700 samples was tested with culture each study period and only culture positive samples were further tested for antimicrobial susceptibility. The latter was performed by disk diffusion according to EUCAST 2018 guidelines to test for susceptibility for erythromycin, penicillin, tetracycline, and cotrimoxazole – if disc diffusion showed resistance for penicillin, the minimum inhibitory concentration (MIC) was determined by E-test (Biomérieux, Craponne, France). A MIC of >0.06 mg/L for penicillin was interpreted as resistant.[7] For the other three antibiotics tested, both I and S categories were interpreted as susceptible.

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 pretreatment[7]. *S. pneumoniae, H. influenzae*, and *M. catarrhalis* DNA was detected by performing real-time PCR targeting the genes *lytA*,[12] *P6*,[13] and *copB* [1], respectively. Samples were classified as positive when C_T values were ≤35.

Molecular serotyping of S. pneumoniae

Molecular serotyping by real-time PCR was performed on all *lytA*-positive samples by using previously published primers and probes for all serotypes included in PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9 V, 14, 18C, 19A, 19F, 23F).[14–16] For serogroup 6, the genes *wciP* and *wciN* were analyzed by three real-time PCRs targeting, respectively, the single nucleotide polymorphism at codon 195[14,15] and the *wciN* gene.[16] This approach allows to

detect serotype 6A and 6C separately. Samples were pooled and screened for 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.

Statistical analysis

Sample size was calculated using the R-package 'power'[7] to achieve 80% power to detect a 4% difference in carriage prevalence of three pneumococcal vaccine serotypes (19A, 6A, 3) over three years (2018–2019, 2019–2020, 2020–2021) (with type I error, α = 0.05). We considered samples *S. pneumoniae*-positive if *S. pneumoniae* was detected either in culture of with molecular analysis/PCR. Serotypes were determined by performing culture based and/or by real-time PCR based analysis (PCV13 vaccine serotypes only).

Here, we describe the results for year 4–6 whereas year 1–3 results are added as reference.[7] The results for the six-year time period (year 1–6) are presented descriptive using tabulations and frequency plots, whereas a statistical analysis was done only for year 4–6. The Chi-Squared (Chi^2) Test, performed in GraphPad Prism version 10.3.1, was used to assess significant differences in carriage frequency of pneumococcal serotypes and nasopharyngeal co-colonizers over time (year 4–6) (α = 0.05).

Results

Over a collection period of three years, a total of 2,911 nasopharyngeal samples obtained from healthy children attending DCCs were included. Nasopharyngeal samples were analyzed by conventional culture (n = 2,046) and/or real-time PCR (n = 2,726). A total of 170 samples were not analyzed by any method because the target number of samples had been surpassed in the geographical area where they were collected, which happened mainly in year 4. These children were not taken into account in the analysis.

Main aim: changes in carriage prevalence of PCV13-vaccine and non-vaccine strains after the PCV10 to PCV13 program switch (2019)

During the study period, the carriage of *S. pneumoniae* remained high with an overall carriage prevalence of 77.1%. A downward trend was observed from year 4 (2018–2019) to year 6 (2020–2021), but the difference was non-significant (p = .063) (Table 1).

19A was the most prevalent vaccine serotype but decreased significantly

The carriage frequency of PCV13 serotypes decreased significantly among *S. pneumoniae* positive samples (n = 2,114) from 19.4% in 2018–2019 to 9.9% in 2020–2021 (p < .001) (Figure 1a). This was mainly due to the significant decrease in the carriage of serotype 19A of which the frequency halved from the fourth to the sixth study year (Table 2). The proportion of children who were vaccinated with PCV13, which covers serotype 19A, increased from year 4 to year 6 (Figure 2, PCV13-only or mixed schedule). For the other present PCV13 serotypes 3 and 19F, a low carriage rate could be observed, with an overall prevalence around 1% for both serotypes. There was, however, an increase in the overall carriage of serotype 3 compared to the overall carriage prevalence in year 1–3.

Among non-vaccine strains, no changes were obvious except serotype 6C

During the PCV10 period (2016–2019), the vaccine-related serotype 6C carriage frequency went up five times. In the next period, carriage of serotype 6C decreased significantly from the fifth to the sixth study year (p = .01).

Table 1. Carriage of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* in children attending daycare centers (DCC) in Belgium.

	Year 1–3* 2016–2018 n = 2818	Year 4 2018–1019 n = 854	Year 5 2019–2020 n = 982	Year 6 2020–2021 n = 905	p-Value** (Chi^2)
S. pneumoniae	2,243; 79.6%	680; 79.6%	756; 77.0%	678; 74.9%	.06
H. influenzae	2,605; 92.4%	779; 92.8%	889; 90.5%	785; 86.7%	**<.001**
M. catarrhalis	2,585; 91.7%	756; 90.1%	906; 92.3%	850; 93.9%	**.01**
S. aureus	122; 4.3%	29; 4.3%	46; 6.7%	54; 7.9%	**.02**
S. pneumoniae–H. influenzae–M. catarrhalis	1,991; 70.7%	593; 70.7%	669; 68.1%	581; 64.2%	**.01**
H. influenzae–M. catarrhalis	2,404; 85.3%	711; 84.7%	831; 84.6%	743; 82.1%	.23
S. pneumoniae–H. influenzae	2,097; 74.4%	624; 74.4%	697; 71.0%	598; 66.1%	**<.001**
S. pneumoniae–M. catarrhalis	2,123; 75.3%	623; 74.3%	715; 72.8%	654; 72.3%	.63

Numbers and proportions of children attending daycare centers and positive for at least one of the three pathogens studied are shown.
n = number of samples analyzed by PCR and/or culture per season.
Number of samples analyzed PCR and/or culture: year 4: 854; year 5: 982; year 6: 905.
Number of samples analyzed by PCR: year 4: 839; year 5: 982; year 6: 905.
Number of samples analyzed by culture: year 4: 673; year 5: 691; year 6: 682.
The presence of S. *pneumoniae* was determined by culture and/or PCR, whereas *S. aureus* was assessed by culture. Carriage of *H. influenzae*, *M. catarrhalis* and co-carriage of *S. pneumoniae* and/or *H. influenzae* and/or *M. catarrhalis* was based on PCR results only.
The second switch from PCV10 to PCV13 happened after the collection period of year 4. The COVID-19 pandemic started during year 5. So, before COVID-19 pandemic can be considered as year 4 and year 5 (885 samples collected until March 11, 2020); During COVID-19 pandemic can be considered as year 5 (97 samples collected from March 11, 2020) and year 6.
*Year 1–3 is added as reference.
**Chi^2 is calculated over three years (year 4, 5, and 6). Significant p-values <0.05 are shown in bold, which we interpreted as a cutoff for significance.

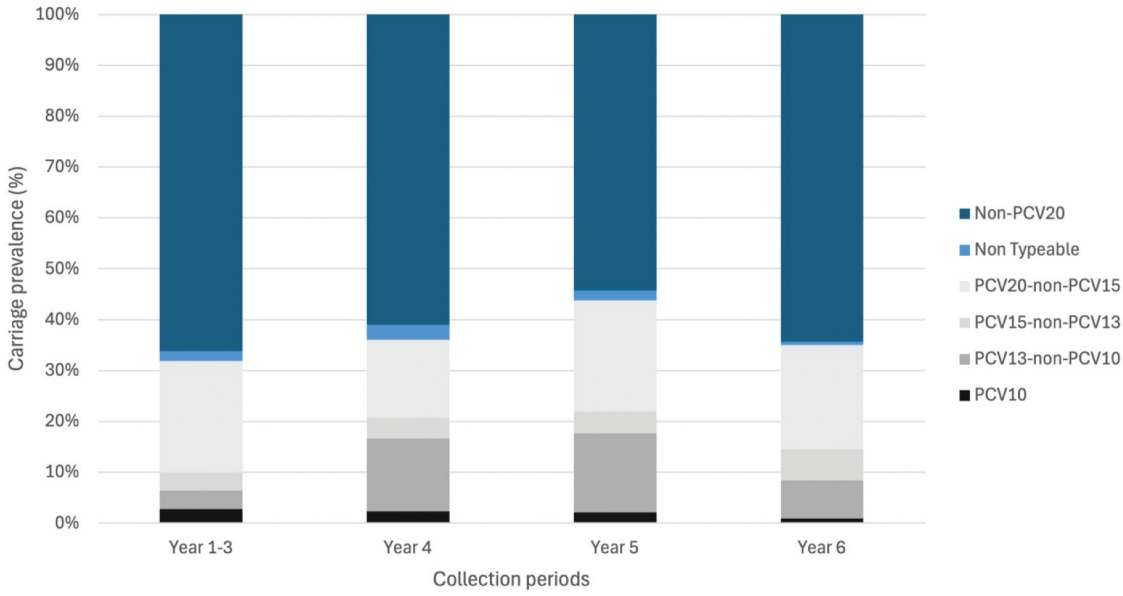

Figure 1(a). Serotype specific carriage frequency among culture positive samples by vaccine-type per study year: PCV10, PCV13-non-PCV10, PCV15-non-PCV13, PCV20-non-PCV15, non typeable and non-PCV20 serotypes. Serotype frequency of PCV10, PCV13-non-PCV10, PCV15-non-PCV13, PCV20-non-PCV15, non typeable, and non-PCV20 serotypes in children attending daycare centers who carried pneumococci. Carriers were considered to be vaccine type carriers if they were positive for a vaccine type and a non-vaccine type carriers if only a non-vaccine type could be detected. *n* = culture positive samples for *S. pneumococcus*. *n* (year 1–3) = 1,883; *n* (year 4) = 474; *n* (year 5) = 470; *n* (year 6) = 463. PCV10 serotypes: 1, 4, 5, 6B, 7F, 9 V, 14, 18C, 19A, 19F, 23F. PCV13-non-PCV10 serotypes: 3, 6A, 19A. PCV15-non-PCV13 serotypes: 22F, 33F. PCV20-non-PCV15 serotypes: 8, 10A, 11A, 12F, 15B.

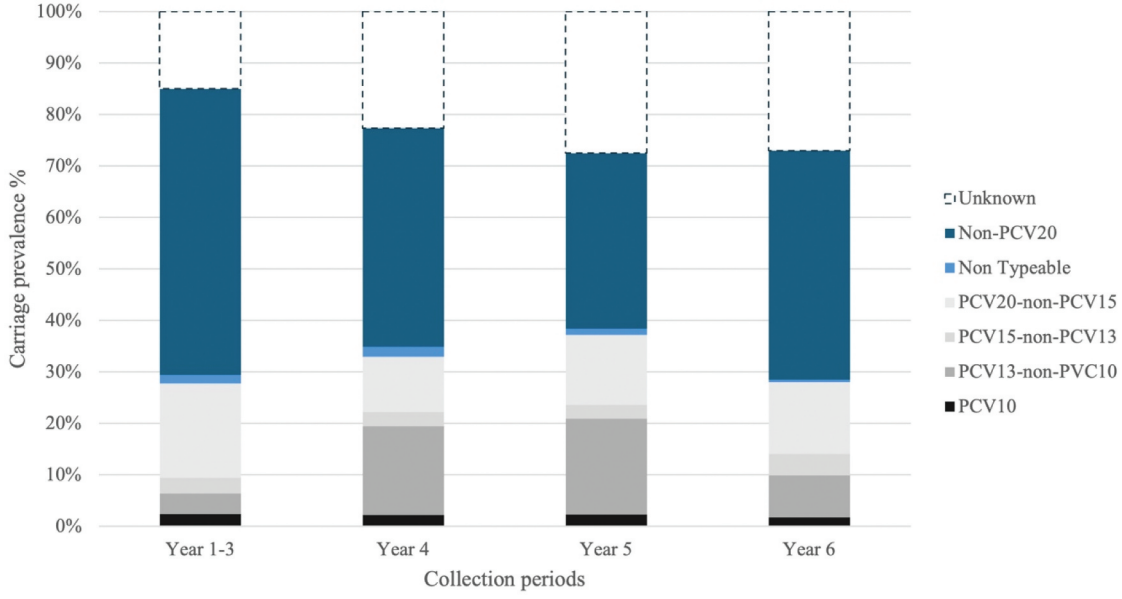

Figure 1(b). Serotype specific carriage frequency among pneumococcal carriers by vaccine-type per study year, culture and/or PCR based for PCV10 and PCV13-non-PCV10 serotypes, and culture based for PCV15-non-PCV13, PCV20-non-PCV15, non typeable and non-PCV20 serotypes. Serotype frequency of PCV10, PCV13-non-PCV10, PCV15-non-PCV13, PCV20-non-PCV15, non typeable, and non-PCV20 serotypes in children attending daycare centers who carried pneumococci. Carriers were considered to be vaccine type carriers if they were positive for one vaccine type and a non-vaccine type carriers if we detected no vaccine type. *n* = culture and/or PCR positive samples for *S. pneumococcus*. *n*(year 1–3) = 2243; *n*(year 4) = 680; *n*(year 5) = 756; *n*(year 6) = 678. PCV10 serotypes: 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F. PCV13-non-PCV10 serotypes: 3, 6A, 19A. PCV15-non-PCV13 serotypes: 22F, 33F. PCV20-non-PCV15 serotypes: 8, 10A, 11A, 12F, 15B. Unknown: Samples positive for pneumococcus by *lytA*-PCR but negative for PCV13 serotypes by serotype-specific PCR, and not analyzed by culture. As serotype-specific PCR was limited to PCV13 serotypes, it remains uncertain which serotypes are carried.

Table 2. Carriage frequency of PCV 13 serotypes and non-vaccine serotype 6C in culture and/or *lytA* positive samples.

	Year 1–3* 2016–2018 *n* = 2,243	Year 4 2018–2019 *n* = 680	Year 5 2019–2020 *n* = 756	Year 6 2020–2021 *n* = 678	Overall** *n* = 2,114
Serotype 1	1; 0.0%	0; 0.0%	0; 0.0%	0; 0.0%	0; 0.0%
Serotype 3	7; 0.3%	9; 1.3%	14; 1.9%	7; 1.0%	30: 1.4%
Serotype 4	0; 0.0%	0; 0.0%	0; 0.0%	0; 0.0%	0; 0.0%
Serotype 5	0; 0.0%	0; 0.0%	0; 0.0%	1: 0.1%	1; <0.1%
Serotype 6A	2; 0.1%	3; 0.4%	1; 0.1%	0; 0.0%	4; 0.2%
Serotype 6B	0; 0.0%	2; 0.3%	0; 0.0%	0; 0.0%	2; <0.1%
Serotype 7F	0; 0.0%	3; 0.4%	2; 0.3%	3; 0.4%	8; 0.4%
Serotype 9V	0; 0.0%	1; 0.1%	1; 0.1%	0; 0.0%	2; <0.1%
Serotype 14	5; 0.2%	0; 0.0%	3; 0.4%	1; 0.1%	4; 0.2%
Serotype 18C	1; 0.0%	0; 0.0%	0; 0.0%	0; 0.0%	0; 0.0%
Serotype 19A	81; 3.6%	105; 15.4%	126; 16.7%	48; 7.1%	279; 13.2%
Serotype 19F	43; 1.9%	6; 0.9%	11; 1.5%	7; 1.0%	24; 1.1%
Serotype 23F	3; 0.1%	3; 0.4%	0; 0.0%	0; 0.0%	3; 0.1%
Serotype 6C	69; 3.1%	106; 15.6%	156; 20.6%	105; 15.5%	367; 17.4%

Carriage frequency of PCV13 serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) and non-vaccine serotype 6C in children attending daycare centers during the period 2018–2019, 2019–2020, and 2020–2021. The second switch from PCV10 to PCV13 happened after the collection period of year 4. *n* = number of culture and/or *lytA* positive samples.
*Year 1–3 is added as reference.
**Overall: for years 4–6.

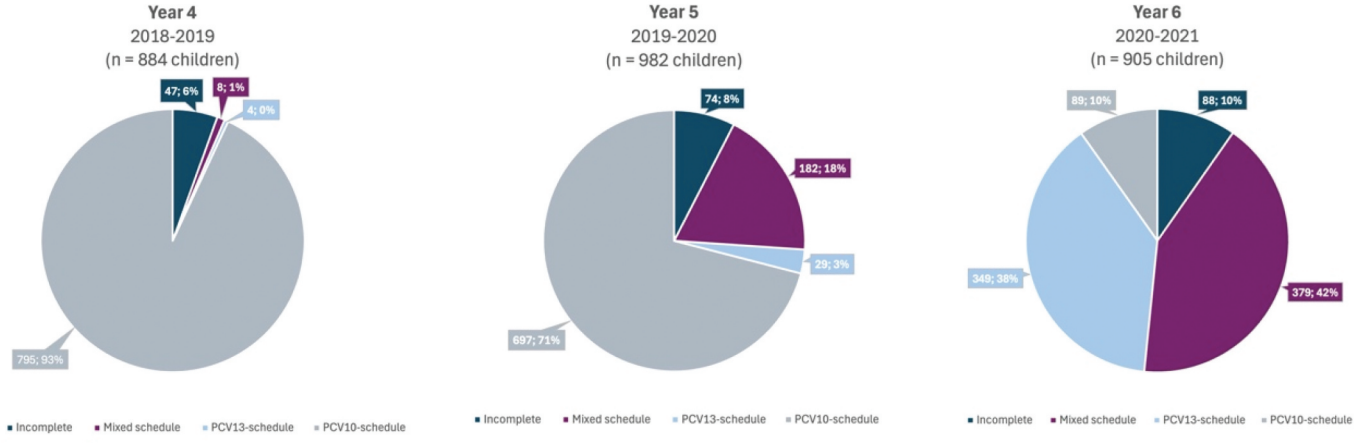

Figure 2. Vaccination status of healthy children 6–30 months in day care centers (2018–2019 *n* = 854; 2019–2020 *n* = 982; 2020–2021 *n* = 905). Percentage of children receiving a PCV13-only schedule, a PCV10-only schedule, a mixed schedule or incomplete vaccination (meaning: number of doses was not age-appropriate). A child was considered fully vaccinated against pneumococcal disease if it had received at least 2 doses up to 12 months of age or at least 3 doses if its age was 12 months or above. Preterm infants were considered fully vaccinated against pneumococcal disease if they had received at least 3 doses up to 12 months of age or at least 4 doses if their age was 12 months or above. The vaccine switch from PCV10 to PCV13 occurred in summer 2019.

Secondary aims: comparing culture-based with PCR-based detection of S. pneumoniae, antimicrobial susceptibility of carried pneumococci, and prevalence of other nasopharyngeal colonizers over the study period

Combining both culture- and PCR-based serotyping slightly increases the detection rate of specific serotypes

Over the three-year study period, a total of 2,031 samples were analyzed with both PCR and culture (Table 3). With PCR, 77 PCV13 serotypes and 45 6C serotypes were additionally found that were not detected with culture. With culture, 5 PCV13 serotypes and 1 6C serotype were additionally found that were not detected with PCR in the same samples.

Frequency of pneumococcal resistance to penicillin increased significantly

The proportion of pneumococcal strains that were susceptible to all four antibiotics tested (penicillin, tetracycline, erythromycin, and cotrimoxazole) remained stable from year 4 to year 6 (year 4: 256/474; 54.0%, year 5: 275/470; 58.5%, year 6: 247/463; 53.3% – $p = .22$). Similarly, no significant changes in resistance either to a single or to multiple antibiotics were observed over the study period (resistance to a single antibiotic – $p = .94$; resistance to multiple antibiotics – $p = .12$) (Figure 3a).

Tetracycline and erythromycin resistance rates did not change significantly over the study period (tetracycline – $p = .35$; erythromycin – $p = .22$) (Figure 3b). However, resistance to penicillin did increase significantly over the three study years (year 4: 106/474; 22.4%, year 5: 108/470; 23.0%, year 6: 135/463; 29.2% – $p = .03$). Notably, overall antimicrobial resistance to cotrimoxazole remained stable over the study period (Figure 3b), although there was a significant dip in year 5 ($p = .005$).

Frequency of resistance to penicillin among carried 6C strains increased

As serotypes 19A and 6C showed a consistent carriage trend over time associated with the changes in the vaccination

Table 3. PCV13 serotypes and serotype 6C with culture versus molecular analysis over the three-year study period.

Research method	PCR only	PCR and culture			Culture only
	$n = 695$	$n = 2,031$			$n = 15$
SPN detected		PCR only	PCR and culture	Culture only	
Serotype 3	7	11	11	1	0
Serotype 5	0	1	0	0	0
Serotype 6A	1	0	3	0	0
Serotype 6C	97	45	221	1	1
Serotype 7F	0	8	0	0	0
Serotype 9V	0	2	0	0	0
Serotype 14	1	1	2	0	0
Serotype 19A	68	50	160	1	0
Serotype 19F	4	4	16	0	0
Serotype 23F	0	0	0	3	0

PCV13 serotypes and serotype 6C detected with culture and/or molecular analysis (PCR) for samples collected during the period 2018–2019, 2019–2020, and 2020–2021. *n* = number of samples analyzed with either PCR only, culture only, or both PCR and culture. Samples analyzed with PCR and culture may be positive for *S. pneumococcus* with either PCR only, culture only, or with both PCR and culture. SPN: *Streptococcus pneumoniae*.

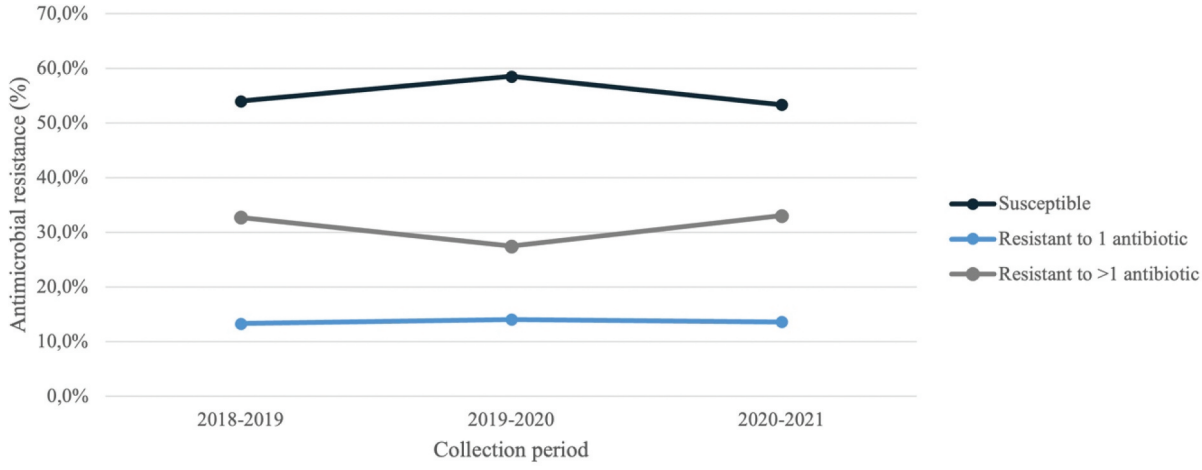

Figure 3(a). Antimicrobial susceptibility profiles to penicillin, tetracycline, erythromycin, and cotrimoxazole of pneumococci carried by healthy children aged 6–30 months. Percentages of cultured pneumococcal strains according to susceptibility to one or multiple tested antibiotics (penicillin, tetracycline, erythromycin, and cotrimoxazole). *n*(year 4) = 474; *n*(year 5) = 470; *n*(year 6) = 463.

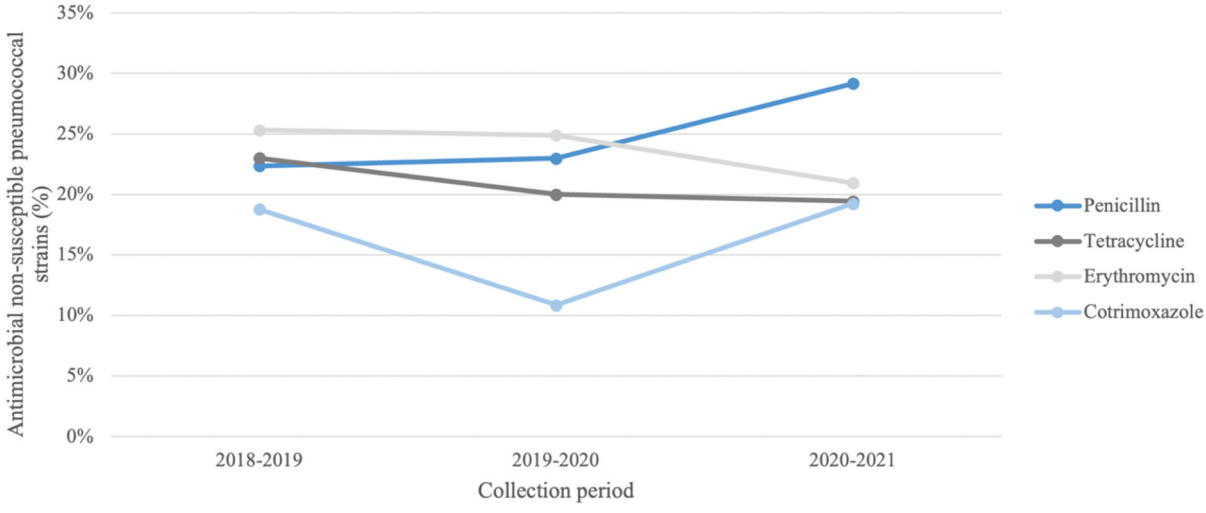

Figure 3(b). Antimicrobial resistance to penicillin, tetracycline, erythromycin, and cotrimoxazole in pneumococcal culture-positive samples. Percentages of cultured pneumococcal strains that are resistant to penicillin, tetracycline, erythromycin, and cotrimoxazole. *n*(year 4) = 474; *n*(year 5) = 470; *n*(year 6) = 463.

program, the antimicrobial resistance of both 19A and 6C strains was studied in more detail (Table 4). For serotype 6C, the resistance to penicillin differed significantly over the three-year study period ($p = .04$), with the lowest frequency in year 4. Serotype 6C strains showed also more often antimicrobial resistance compared to 19A strains except for cotrimoxazole.

Table 4. Antimicrobial resistance to penicillin, tetracycline, erythromycin, cotrimoxazole, and to at least one antibiotic in culture-positive serotypes 19A and 6C strains.

	Serotype 19A				Serotype 6C			
	Year 4 2018–2019 $n = 63$	Year 5 2019–2020 $n = 65$	Year 6 2020–2021 $n = 33$	p-Value*	Year 4 2018–2019 $n = 80$	Year 5 2019–2020 $n = 77$	Year 6 2020–2021 $n = 69$	p-Value*
Penicillin	5; 7.9%	4; 6.2%	4; 12.1%	0.59	12; 15.0%	23; 29.9%	21; 30.4%	**0.04**
Tetracycline	14; 22.2%	11; 16.9%	5; 15.2%	0.63	40; 50.0%	47; 61.0%	43; 62.3%	0.24
Erythromycin	17; 27.0%	10; 15.4%	5; 15.2%	0.19	41; 51.3%	48; 62.3%	45; 65.2%	0.18
Cotrimoxazole	6; 9.5%	2; 3.1%	2; 6.1%	NA**	3; 3.8%	0; 0.0%	4; 5.8%	NA**
At least one antibiotic	22; 34.9%	14; 21.5%	6; 18.2%	0.12	43; 53.8%	49; 63.6%	47; 68.1%	0.18

Numbers and percentages of pneumococcal 19A and 6C strains that are resistant to penicillin, tetracycline, erythromycin, cotrimoxazole, and to at least one antibiotic for three study years. n = number of strains analyzed by culture and positive for 19A or 6C. NA = not applicable.
*Chi^2 is calculated over three years (year 4, 5, and 6). Significant p-values <.05 are shown in bold, which we interpreted as a cutoff for significance.
**At least 20% of the expected values have to be greater than 5. These conditions have not been met, and thus the chi-square could not be calculated.

No significant trends in resistance to tetracycline, erythromycin, or cotrimoxazole were observed over the three study periods (Table 4).

Carriage of H. influenzae *decreased whereas carriage of* M. catarrhalis *and* S. aureus *increased*

As shown in our previous study,[5] *H. influenzae* and *M. catarrhalis* remained the most frequently carried pathogens in our study population, whereas *S. aureus* remained uncommon (Table 1). During the three-year study period, a significant decrease in the *H. influenzae* carriage and a significant increase in *M. catarrhalis* and in the rarely present *S. aureus* were seen (Table 1). Co-carriage of *M. catarrhalis* with *H. influenzae* (83.4%) was most frequent and they both co-occurred with *S. pneumoniae* at similar rates, 70.0% with *H. influenzae*, and 72.7% with *M. catarrhalis*.

Looking in detail to the *H. influenzae carriage*, the significant carriage decrease was only found in children with *H. influenzae* strains that were co-carrying *S. pneumoniae*. The proportion of children carrying only *H. influenzae* remained stable over the three-year study period (2018–2019: 155/839, 18.5%; 2019–2020: 189/982, 19.2%; 2020–2021: 187/905, 20.7% – $p = .51$).

Comparing samples collected before and during the COVID-19 pandemic (2020–2021)

During the three-year study period, a total of 1,739 samples were collected before non-pharmaceutical interventions (NPIs) were introduced to control the COVID-19 pandemic (samples collected until March 11, 2020), were analyzed by either PCR, culture or both. When NPIs were in place (samples collected from March 11, 2020 until June 2021), a total of 1,002 samples were analyzed. For simplicity, we refer to the first period as "before/pre-COVID-19" and to the latter as "during COVID-19." Importantly, the country's first lockdown (March–May 2020) coincided with our year 5 study period, during which daycare centers remained open albeit with only 50% occupancy. By contrast, during the winter of 2020–2021 which coincided with our year 6 study period, the number of children attending DCCs was comparable to previous years, with few restrictions imposed in these settings.[17] However, there were still restrictions on social contacts (e.g. household members), which are likely to have had an effect. Given our hypothesis that NPIs may have influenced overall pathogen carriage and antibiotic prescription rates, we compared carriage rates and antibiotic susceptibility between pre-COVID-19 and during COVID-19 periods.

Overall carriage of *S. pneumoniae* (before COVID-19: 79.0%; during COVID-19: 73.9% – $p = .002$), *H. influenzae* (before COVID-19: 92.3%; during COVID-19: 85.9% – $p < .0001$), and co-carriage of *S. pneumoniae* with *H. influenzae* (before COVID-19: 73.6%; during COVID-19: 64.9% – $p < .0001$) and jointly with *M. catarrhalis* and *H. influenzae* (before COVID-19: 70.2%; during COVID-19: 63.1% – $p < .0001$) decreased significantly compared to pre-COVID-19. An opposite trend was observed for the overall carriage of *M. catarrhalis* (before COVID-19: 91.3%; during COVID-19: 93.6% – $p = .03$) and *S. aureus* (before COVID-19: 5.4%; during COVID-19: 7.8% – $p = .03$).

Antimicrobial resistance to penicillin (before: 22.7%; during: 28.7% – $p = .01$) increased significantly during the COVID-19 period. In contrast, no significant changes were found in resistance to tetracycline (before: 20.9%; during: 21.0% – $p = .97$), erythromycin (before: 24.8%; during 22.2% – $p = .27$), cotrimoxazole (before: 15.1%, during 18.3% – $p = .13$), or resistance to at least one antibiotic (before: 43.9%; during 46.6% – $p = .33$).

Year 6 was the only study period where all samples were collected during COVID-19. This allowed us to compare children who had experienced a pre-COVID-19 context since their birth ($n = 751$ in year 6) with those who had not ($n = 153$ in year 6), with respect to overall carriage and antibiotic susceptibility. Significant differences were seen in the carriage of *M. catarrhalis* (Born before: 94.7%; Born during: 90.2% – $p = .03$) and in the co-carriage patterns of *S. pneumoniae* with *H. influenzae* (Born before: 66.6%; Born during; 62.7% – $p = .001$), *S. pneumoniae* with *M. catarrhalis* (Born before: 72.8%; Born during 64.7% – $p < .001$), and finally *S. pneumoniae* with *H. influenzae* and *M. catarrhalis* (Born before 65.0%; Born during 60.8% – $p = .001$). No significant differences in antimicrobial resistance were observed between children born and during the COVID-19 pandemic.

Discussion

Already in the second year after the back-switch to PCV13 a clear decrease was shown in the carriage of PCV13 vaccine serotypes in samples obtained from children attending day-care centers. This decrease was mainly caused by the decrease in prevalence of PCV13-non-PCV10 vaccine serotype 19A. In

the present study, also a clear decreasing trend was observed in the prevalence of the vaccine-related serotype 6C from year 5 to year 6.

Previously, our group already reported the significant increase in the carriage of the PCV13-non-PCV10 serotype 19A as well as the increase in the carriage of the vaccine-related serotype 6C in children attending daycare centers during the PCV10 period (2016–2019).[5] This increase was likely due to the switch in the Belgian pediatric vaccination program from PCV13 to PCV10 in 2016. The Belgian Superior Health Council recommended preferential use PCV13 in 2019.[18] After implementation of this switch in 2019, we observed a decreasing trend in the prevalence of serotype 19A in the present study. In Belgium, also a reduction was observed in serotype 19A caused IPD cases in children under two years of age in 2021 (27.0% in 2018; 39.4% in 2019; 43.8% in 2020 and 14.0% in 2021).[19–21] These findings were similar as in European countries and the USA, where the inclusion of the PCV13 vaccine in the vaccination program resulted in a decline in the percentage of PCV13 vaccine serotype strains, especially in serotype 19A strains.[22–25]

The other serotype that substantially increased during the PCV10 period was serotype 6C. However, in the present study, we observed a decreasing trend in serotype 6C prevalence from year 5 to year 6. Our serotype comparisons showed that the switch from PCV10 to PCV13 was more effective in reducing 19A than serotype 6C carriage. Contrary to our carriage data, routine IPD surveillance showed similar trends for isolates containing serotype 6C and 19A (for serotype 6C 0.7% in 2018; 2.1% in 2019; 3.8% in 2020 and 0.0% in 2021) in children under two years of age over the consecutive periods.[19,20] In other European studies in children, effective protection against serotype 6C IPD was also found by vaccination with PCV13,[26] but not with PCV10.[26]

In the present study, the resistance of pneumococcal strains to penicillin increased significantly over the study period. The antimicrobial resistance to penicillin in Belgian IPD cases was also highest in 2021 (18.4%).[19,20] Different studies in the literature also show a high prevalence of resistance to penicillin in pneumococcal strains, especially in serotype 19A strains.[26–28] The likelihood of resistance to a specific antibiotic in a pneumococcal strain is particularly associated with longer carriage duration, greater resistance to neutrophil-mediated killing, the larger capsule size, and the metabolic efficiency for capsule production.[28]

Overall *S. pneumoniae* carriage remained stable over the three study years, while *H. influenzae* carriage decreased and conversely *M. catarrhalis* carriage increased significantly. Subsequently, again a high co-colonization rate was seen between *S. pneumoniae* and *M. catarrhalis* or *H. influenzae*, which is in line with previous reports.[5,6]

However, when comparing samples taken before versus during the COVID-19 period, we observed a decrease in the carriage of the analyzed pathogens including *S. pneumoniae*, except for *M. catarrhalis* and *S. aureus*. This finding contrasts with a previous study of our group when the COVID-19 pandemic just emerged (July 2020–June 2021)[17] and where no difference was observed in the carriage of *S. pneumoniae*. Also, in other studies, no difference was seen in pneumococcal carriage proportions in the first year after the start of the COVID-19 pandemic (winter season 2020–2021), while there was a decrease in IPD cases as well as in the circulation of different respiratory viruses,[29,30] suggesting that the decline in IPD and respiratory viruses during the initial year of COVID-19 are related with each other.[31] Future research may investigate the relationship between the (non)-circulation of viruses and pneumococcal carriage. Up to our knowledge, there is no other study showing a decrease in the carriage of *S. pneumoniae* in the COVID-19 period.

Next to the unique context including both a switch and a back-switch in the vaccine program, a major strength of this study protocol is the quick processing of the samples. Samples from DCC infants are transported immediately (cooled transport 2–8°C) and stored frozen (−80°C) within 8 hours after collection. Within 24 months, both culture and PCR methods are performed. An annual contamination check is performed on blank samples. Finally, PCR can detect both living and non-living germs, making its results less sensitive to eventual differences in transport conditions than culture.

However, there are some limitations to this study. The sample size was calculated to detect a change in the proportion of serotype 19A prevalence, so smaller differences in carriage of other serotypes may have been missed. Furthermore, the number of samples assessed for the presence of vaccine serotype carriage (all *lytA* positives) is higher than for the presence of non-vaccine serotype carriage, which is determined by culture only except from serotype 6C (Figure 1b). Therefore, the probability of detecting a significant trend in carriage of non-vaccine serotypes over time is lower. The number of antibiotics against which resistance of pneumococcal strains is tested, is limited. Considering the two-year age difference between the youngest and oldest children included, there is a mixture of different vaccine statuses; however, the age repartition of participating children is stable over the collection periods. Participation bias cannot be excluded since recruitment is voluntary, but the socio-demographic profile of participants was found stable over time. However, the results are valid only for children attending daycare centers which means 40% of the Belgian children[32] are not included. Finally, as this is an observational study, it can only show associations in time but no causal relationship. The COVID-19 pandemic and especially the NPIs may play a part in some of our findings, but our comparisons between pre-COVID-19 and during COVID-19 results do not suggest a major impact on our main findings.

To conclude, a sustained high carriage of *Streptococcus pneumoniae* was observed in combination with a decreasing proportion of the PCV13-non-PCV10 serotype 19A after the second switch to PCV13 in 2019 in Belgium. Both the clear trend and the association in time indicate the vaccine-switch as main cause of these changes. Continued surveillance will demonstrate whether the use of PCV13 will result in a further decrease of serotype 19A and whether a clear continued trend in reduced carriage of serotype 6C can be observed. Our study was the first to show a decrease of *S. pneumoniae* carriage during the COVID-19 period.

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