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Serotype distribution and antimicrobial susceptibilities of nasopharyngeal isolates of *Streptococcus pneumoniae* from healthy children in the 13-valent pneumococcal conjugate vaccine era

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ABSTRACT

Few epidemiological data are available since the introduction of 13-valent pneumococcal vaccine (PCV13) in 2010. We conducted a cross-sectional study to estimate the prevalence of *Streptococcus pneumoniae* (SP) nasopharyngeal carriage in healthy Italian infants and young children and to evaluate the impact of PCV13 on pneumococcal colonization. In the trimester September–December 2011 nasopharyngeal swabs were collected from healthy children aged 3–59 months presenting for routine well careat 16 primary care pediatricians in Milan.

SP carriage isolates were serotyped and tested for antimicrobial resistance using EUCAST breakpoints. Among 1250 enrolled children, 618 had received at least 1 dose of PCV13, 292 at least 1 dose of PCV7, 94 a combination of the two vaccines and 246 were not vaccinated. The prevalence of SP carriage was 27% (95% confidence interval [CI] 25–30).

At multivariable analysis, age ≥ 25 months (prevalence ratio [PR] = 0.74) and use of antibiotics in the previous 3 months (PR = 0.67) were associated with lower SP carriage prevalence. Having siblings (PR = 1.79 for 1 sibling and PR = 2.23 for ≥2 siblings), day-care attendance (PR = 2.27) and respiratory tract infections in the previous 3 months (PR = 1.39) were associated with higher SP carriage prevalence.

The immunization status for SP was not associated with SP carriage at univariable or at multivariable analysis.

Abbreviations: CI, confidence interval; IPD, invasive pneumococcal diseases; MIC, minimal inhibitory concentration; MDR, multidrug resistance; NC, nasopharyngeal carriage; NVS, non vaccine serotypes; PCV7, pneumococcal 7-valent vaccine; PCV13, pneumococcal 13-valent vaccine; PR, prevalence ratio; SP, *Streptococcus pneumoniae*.

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The most common carriage isolates were 6C, 19A and 23A. The prevalence of the six additional PCV13 serotypes carriage in children appropriately vaccinated with PCV13 was lower than in children appropriately vaccinated with PCV7 (0 vs. 0.060); the greater reduction in prevalence of carriage was observed for serotype 19A (0 vs. 0.041). Serotype 6C was the most common drug-resistant serotype (17.2%).
Further epidemiological studies are needed to assess changes in circulating SP serotypes following the large-scale introduction of PCV13.

1. Introduction

Streptococcus *pneumoniae* (SP) is a major cause of respiratory and invasive disease worldwide, particularly in infants and young children, causing approximately 11% of all deaths in children aged 1–59 months [1]. It is well known that the nasopharynx of children serves as a natural reservoir from where it can give rise to invasive disease, serves as the source of person to person transmission and represents the environment in which dynamical evolution of SP occurs. Nasopharyngeal isolates may represent an indicator of invasive disease, antibiotic resistance profiles, and potential vaccine coverage. However, certain serotypes and genotypes seem to cause higher rates of invasive disease when corrected for prevalence of nasopharyngeal colonization [2]. Therefore, continuous surveillance of invasive pneumococcal diseases (IPDs) and colonization isolates is warranted in countries where large-scale pneumococcal vaccination is initiated.

In many industrialized countries, the widespread use of the 7-valent pneumococcal conjugate vaccine (PCV7) in children has led to a dramatic decline in PCV7-serotype IPDs, not only in vaccinated children but also in unvaccinated persons of all ages [3–5]. Intriguingly, shifts toward non-PCV7 serotypes IPDs have occurred, driving the rise of 19A; noteworthy, with the widespread use of antibiotics the antimicrobial resistance of this strain is evolving [6–13]. Based on the changing epidemiological picture, a new 13-valent pneumococcal conjugate vaccine (PCV13) including all PCV7 serotypes and six additional serotypes (1, 3, 5, 6A, 7F, and 19A) was developed [14,15]. Concerns are rising regarding a new selective pressure, that may alter the current equilibrium among pneumococcal carriage isolates, and lead to a non-PCV13 serotype shift.

In Italy the PCV7 was firstly recommended for the immunization of children in 2005; in summer 2010 it was replaced by the PCV13. Thus, from 2010 infants' immunization with 3 doses of PCV13 at 3, 5–6, and 11–12 months or with 2 doses in children in the second year of life is recommended; while in children aged 2–5 years a single dose of PCV13 is administered. During the transition period, switching from PCV7 to PCV13 was recommended at any point in the schedule to complete the immunization series. Moreover, a serotype catch-up was recommended for all children who had been previously appropriately vaccinated for age with PCV7.

To date, data regarding SP circulation in Italian children are limited [16]. Moreover, few studies are available to evaluate the nasopharyngeal carriage of SP in healthy children who had received PCV13 [17]. Given the crucial importance of SP surveillance we seek to evaluate the prevalence, serotype distribution, and antibiotic susceptibility of nasopharyngeal strains of SP following the introduction of PCV13. The study was carried out in a population of healthy children aged 3–59 months resident in the Milan metropolitan area (Lombardy, Northern Italy), Demographic and behavioral factors associated with SP carriage in this population were also investigated.

2. Materials and methods

2.1. Study design

We performed a cross-sectional study aimed to assess the prevalence, serotype distribution and antibiotic susceptibility of nasopharyngeal SP carriage isolates in Italian healthy children aged 3–59 months living in the Milan metropolitan area.

The study was performed on a convenience sample of 16 primary care pediatricians affiliated to the Italian National Health System enrolled by word of mouth by the study principal investigator (PI). An anonymous coded list of potentially eligible children was provided by each primary care pediatrician and sent to the study PI. In this list, the pediatrician code, an arbitrary patient number and the gender and birthdate of each child were included.

Healthy children aged 3–59 months were eligible for the study. Exclusion criteria were: malformation or trauma of the nasopharynx, fever, acute respiratory tract infection, immunological disease, neoplastic disease, renal disease, cardiac disease, hematological disease, cystic fibrosis, bronchodysplasia and Down's syndrome. To avoid the bias of studying siblings living in the same environment, only one child per family was recruited.

Given the current epidemiological data, the prevalence of SP nasopharyngeal carriage was expected as low as 0.087 [18]. One thousand two hundred children were needed to detect such prevalence with an exact 95% confidence interval [95% CI 0.071–0.104]. Assuming a non-response rate of 10%, we calculated that 1320 children had to be enrolled.

The study protocol was approved by the Luigi Sacco Hospital Ethical Committee. Written informed consent was obtained from the parents or legal guardians of the children.

2.2. Sociodemographic and immunization data collection

Data on age, gender, ethnic group, day care attendance, number of siblings, passive smoking, respiratory tract infection in the preceding 3 months, antimicrobial use in the previous 3 months, antimicrobial use in the preceding week was collected. Records of children vaccination states were obtained from local Public Health Authorities. Children were considered appropriately vaccinated for age if they had received all the recommended doses (2 + 1 doses), or a catch-up dose at >1 year of age, according to the Italian Guidelines. Children were considered incompletely vaccinated for age if they did not complete the schedule for age.

2.3. Microbiologic processing

2.3.1. Sample collection

Nasopharyngeal swabs were collected from children by trained study personnel with a nylon flocked flexible sterile Copan Eswab (immersed in liquid Amies Transport Medium) according to the World Health Organization standard procedures. Specimens were sent to Regional Reference Laboratory for invasive diseases within three hours and immediately processed, or stored at 4–8 °C and plated within 48 h [19].

2.3.2. Bacterial identification and serotyping

All swabs were plated on Columbia horse blood agar and on Columbia horse blood agar containing colistin and nalidixic acid. Agar plates were incubated overnight at 35 °C in air with 5% CO_2. The haemolytic suspected pneumococcal colonies were plated on Columbia horse blood agar, incubated overnight at 35 °C in air with 5% CO_2. The day after, the pneumococcal colonies were identified

by Gram staining, optochin sensitivity and bile solubility testing. Serogrouping was performed on each carriage isolate using a commercial kit for latex agglutination (Pneumotest latex kit, Statens Serum Institut, Copenhagen, Denmark). The agglutination kit contains latex particles coated with rabbit antibodies reacting with specific pneumococcal capsular polysaccharide; the identification of pneumococcal serogroups follows a checkerboard system after agglutination in 14 pool suspensions. The serotype was determined by the capsular reaction test (Quellung reaction) using specific antisera (Statens Serum Institut, Copenhagen, Denmark). A suspension of the organism was prepared in saline solution 0.9% from well isolated colonies grown on sheep blood agar plates for 18–24 h in 5% CO_2 at 35 °C. One drop of this suspension was mixed with 1 drop of antiserum and after incubation at room temperature for 10 min, examined at 400× magnification. Visible evidence of capsular swelling with specific antisera and a positive Quellung reaction, was considered positive tests.

2.3.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of carriage isolates against penicillin G, erythromycin, chloramphenicol, tetracycline, vancomycin, levofloxacin, ceftriaxone, linezolid and trimethoprim-sulfamethoxazole was performed using the *E* test method (AB Biodisk, Solana, Sweden) on Mueller-Hinton agar + 5% horse blood and 20 mg/L β-NAD (bioMérieux, Italia SPA). Inocula were prepared by suspending pneumococcal colonies to a density that matched a McFarland 0.5, incubated in 5% CO_2 at 35 ± 1 °C for 18 ± 2 h. Minimum inhibitory concentration (MIC) was tested reading zone edges as the point showing no growth viewed from the front of the plate with the lid removed and with reflected light, and determined using the breakpoints from the European Committee on Antimicrobial Susceptibility Testing EUCAST – (breakpoint tables for interpretation of MICs and zone diameters Version 3.0, valid from 2013-01-01). Susceptibility to penicillin G were determined using the breakpoints for meningitis ($S \leq 0.06$ μg/ml; $R > 0.06$ μg/ml) and for non meningitis ($S \leq 0.06$ μg/ml; $R > 2.0$ μg/ml). *S. pneumoniae* ATCC 49619 was used as control in each run as recommended by EUCAST [20]. Nonsusceptibility to ≥3 antibiotic classes was considered multidrug resistance (MDR).

2.4. Statistical analysis

Categorical variables are reported as counts and percentages. Poisson regression was used to estimate prevalence and prevalence ratios [21]. Robust confidence intervals where calculated when asymptotic assumptions were met and exact confidence intervals when such assumptions were not met [21,22]. Poisson regression with robust confidence intervals is equivalent to binomial regression for estimating binary outcomes but there is presently no exact method for binomial regression and this is explains why we used Poisson regression in this study [21–24]. Univariable Poisson regression was used to obtain prevalence ratios (PR) associated with age (3–12 vs. 13–24 vs. 25–39 months), gender (male vs. female), ethnic group (Caucasian vs. non-Caucasian), number of siblings (0 vs. 1 vs. ≥2), indirect smoke (yes vs. no), respiratory tract infection in last 90 days (yes vs. no) and use of antibiotics in last 90 days (yes vs. no). Multivariable Poisson regression was used to calculate PR associated with all these variables. Statistical analysis was performed using Stata version 13.1 (Stata Corp., College Station, TX, US).

3. Results

Of 1320 eligible children, 1312 (99.3%) were recruited. Parents or legal guardians of only 17 children (1.3%) declined the invitation, thus the respondent rate was much higher than expected (98.7 vs. 90.0%).

Table 1
Characteristics of the study subjects.

	N	%
Age (months)		
3–12	561	44.9
13–24	316	25.3
25–59	373	29.8
Gender		
Female	577	46.2
Male	673	53.8
Ethnic group		
Non-Caucasian	169	13.5
Caucasian	1081	86.5
Siblings		
0	614	49.1
1	472	37.8
≥2	164	13.1
Daycare attendance		
No	785	62.8
Yes	465	37.2
Indirect smoke		
No	782	62.6
Yes	468	37.4
Respiratory infections in the previous 90 days (upper and lower tract)		
No	764	61.1
Yes	486	38.9
Antibiotics in the previous 90 days		
No	1169	93.5
Yes	81	6.5
Antibiotics in the previous 7 days		
No	1169	93.5
Yes	81	6.5
PCV uptake by age		
None	246	19.7
PCV7-incompletely vaccinated for age	28	2.2
PCV7-appropriately vaccinated for age	264	21.1
PCV13-incompletely vaccinated for age	447	35.8
PCV13-appropriately vaccinated for age	171	13.7
At least 2 doses in a combination of the two vaccines	94	7.5

A total of 1295 nasopharyngeal specimens were collected between September 5 and December 6, 2011. Forty-five children (3.2%) were excluded because of incomplete data (immunization schedule and/or sociodemographic variables). Through a careful chart review, emerged that these children were "missed and random" and their exclusion was highly unlikely to impact on the study results [25]. Thus, we performed the analysis on 1250 children for which complete data were recorded. The study subjects' characteristics are given in Table 1.

Among 1250 enrolled children, 618 had received at least 1 dose of PCV13, 292 at least 1 dose of PCV7 while 94 a combination of the two vaccines; 246 were not immunized with any PCV. Of the 292 subjects immunized with PCV7, 264 (90.45) were appropriately vaccinated for age; of the 618 subjects immunized with PCV13, 171 (27.7%) were appropriately vaccinated for age (Table 1). Overall 343 subjects had a nasopharyngeal specimen positive for SP, corresponding to a prevalence of 0.27 (95% CI 0.25–0.30). The prevalence was 0.22 (95% CI 0.19–0.26) in infants aged 3–12 months of age, 0.31

Table 2
Univariable and multivariable prevalence ratios for nasopharyngeal carriage of *Streptococcus pneumoniae*.

	Univariable analysis PR			Multivariable analysis PR[a]		
	Point	95% L	95% U	Point	95%L	95% U
13–24 vs. 3–12 months	1.41	1.21	1.76	1.06	0.85	1.32
25–59 vs. 3–12 months	1.43	1.16	1.77	0.74	0.58	0.94
Male gender	0.89	0.75	1.07	0.91	0.77	1.09
Caucasian	1.25	0.93	1.69	1.17	0.88	1.56
1 sibling vs. no sibling	1.94	1.58	2.39	1.79	1.46	2.19
≥2 siblings vs. 0 sibling	2.14	1.66	2.76	2.23	1.72	2.88
Daycare attendance	2.15	1.79	2.57	2.27	1.85	2.80
Indirect smoke	0.99	0.83	1.20	1.04	0.87	1.24
Respiratory tract infections in the previous 90 days	1.41	1.18	1.68	1.39	1.15	1.68
Antibiotics in the previous 90 days	0.92	0.72	1.17	0.67	0.52	0.86
PCV vaccination	1.09	0.86	1.38	1.03	0.82	1.13

PR: prevalence ratio; point: point estimate; 95% L: lower 95% confidence interval; 95% U: upper confidence interval. PR were calculated using Poisson regression with robust confidence interval

[a] All variables considered at univariable analysis were included in multivariable analysis

(95% CI 0.26–0.36) in young children aged 13–24 months and 0.32 (95% CI 0.27–0.37) in children aged 25–59 months.

Table 2 gives the univariable and multivariable prevalence ratios (PR) for SP nasopharyngeal carriage associated with the predictors of interest.

At univariable analysis, age (PR = 1.41 for 13–24 months and PR = 1.43 for 25–59 months vs. 3–12 months), siblings (PR = 1.94 for one sibling and PR = 2.14 for ≥2 siblings vs. no siblings), day-care attendance (PR = 2.15) and respiratory tract infection in the previous 90 days (PR = 1.41) were predictors of nasopharyngeal SP carriage.

At multivariable analysis however, the prevalence of SP carriage was similar in children aged 3–12 and 13–24 months and lower in children aged 25–59 months (PR = 0.74). Having one or more siblings was still positively associated with the prevalence of SP carriage (PR = 1.79 for 1 sibling and PR = 2.23 for ≥2 siblings vs. no siblings) and the same was true for day-care attendance (PR = 2.27) and for respiratory tract infection in the previous 3 months (PR = 1.39). In addition, the use of antibiotics in the previous 3 months was inversely associated with carriage prevalence (PR = 0.67).

The immunization status for SP was not associated with SP carriage at univariable or at multivariable analysis.

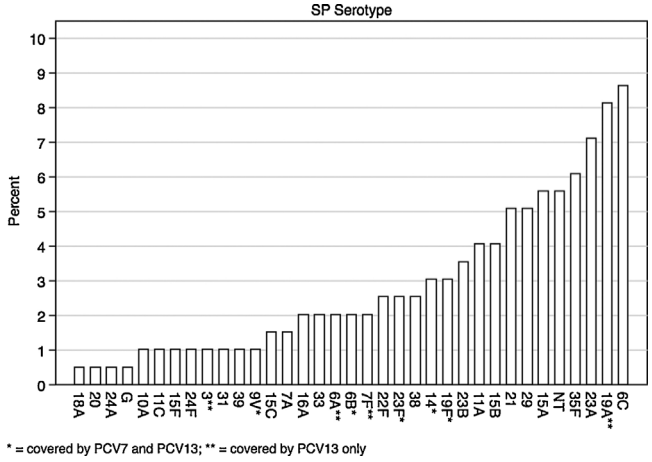

Fig. 1. Serotypes distribution in the whole population.

Table 3
Serotypes by vaccination status. NV, not vaccinated; PCV7, appropriately vaccinated for age with PCV7; PCV13, appropriately vaccinated for age with PCV13.

Serotype	NV		PCV7		PCV13	
	N	%	*N*	%	*N*	%
6C	8	14	7	8	2	5
19A	5	9	11	12	0	0
23A	6	10	4	5	4	11
35F	2	3	5	6	5	13
15A	3	5	6	7	2	5
21	2	3	7	8	1	3
29	2	3	6	7	2	5
15B	2	3	3	3	3	8
11A	1	2	4	5	3	8
23B	1	2	3	3	3	8
19F	3	5	2	2	1	3
14	1	2	4	5	1	3
23F	4	7	0	0	1	3
38	1	2	4	5	0	0
22F	1	2	3	3	1	3
33	1	2	2	2	1	3
16A	1	2	3	3	0	0
6B	3	5	1	1	0	0
6A	3	5	1	1	0	0
7F	1	2	3	3	0	0
15C	1	2	1	1	1	3
7A	2	3	0	0	1	3
11C	1	2	0	0	1	3
10A	0	0	1	1	1	3
39	0	0	1	1	1	3
3	1	2	1	1	0	0
9V	1	2	0	0	1	3
24F	0	0	2	2	0	0
15F	0	0	1	1	1	3
31	0	0	2	2	0	0
24A	0	0	0	0	1	3
20	0	0	1	1	0	0
18A	1	2	0	0	0	0
Total	58	100	89	100	38	100

Fig. 1 gives the frequency distribution of pneumococcal serotypes in all subjects. Table 3 reports the serotype distribution by vaccination status. Fig. 2 contrasts unvaccinated children, appropriately vaccinated for age with PCV7 and PCV13 for the prevalence of SP, PCV7 and PCV13 carriage serotypes and predominant carriage serotypes (6C, 19A, 23A).

The prevalence of SP carriage was 0.256 in unvaccinated children, 0.340 in children appropriately vaccinated with PCV7, 0.257 in children appropriately vaccinated with PCV13. The prevalence of PCV7 serotypes carriage was 0.048 in unvaccinated children, 0.026 in children appropriately vaccinated with PCV7, 0.023 in children appropriately vaccinated with PCV13. The prevalence of PCV13-specific serotypes was 0.040 in unvaccinated children, 0.060 in children appropriately vaccinated with PCV7, 0 in children appropriately vaccinated with PCV13. The prevalence of 6C serotype was 0.032 in unvaccinated children, 0.026 in children appropriately vaccinated with PCV7, 0.011 in children appropriately vaccinated with PCV13. The prevalence of 19A serotype was 0.020 in unvaccinated children, 0.041 in children appropriately vaccinated with PCV7, 0 in children appropriately vaccinated with PCV13. The prevalence of 23A serotype was 0.024 in unvaccinated children, 0.015 in children appropriately vaccinated with PCV7, and 0.023 in children appropriately vaccinated with PCV13. The number of positive outcomes was enough to perform a formal between-PCV comparison only for the prevalence SP carriage. After adjustment for multiple comparisons (Bonferroni's correction), such differences were however not statistically significant (data not shown).

In children immunized with PCV13, the prevalence of PCV13-specific serotypes carriage was 0 in appropriately vaccinated vs. 0.024 in incompletely vaccinated for age subjects (PR = 0.18, 95% exact CI 0–1.166).

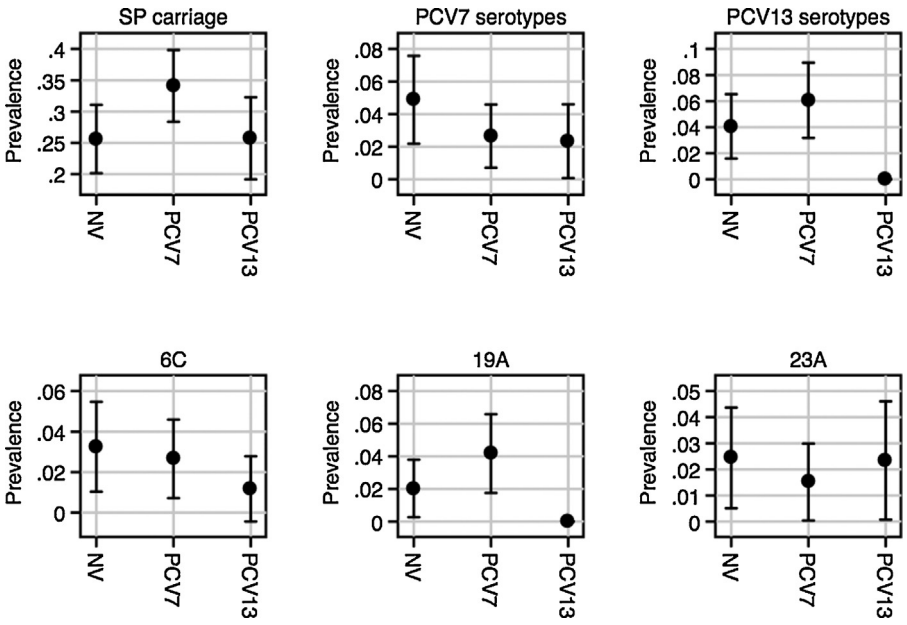

Fig. 2. Prevalence of SP carriage, PCV7, PCV13 and the most common isolated serotypes according to the vaccine status. NV, not vaccinated; PCV7, appropriately vaccinated for age with PCV7; PCV13, appropriately vaccinated for age with PCV13.

The antimicrobial susceptibilities and the MICs of the isolated SP ($n = 343$) are reported in Table 4.

Twenty-nine percent of carriage isolates had intermediate resistance to penicillin and 1.5% was fully resistant. Thirty-five percent and 26% of carriage isolates were resistant to erythromycin and tetracycline, respectively. Resistance to other antibiotics was uncommon. Table 5 shows antibiotic susceptibility and MICs 50/90 of carriage isolates to the common used antibiotics, grouped by vaccine serotypes.

A total of 58/343 (16.9%) carriage isolates showed MDR. Of 58 MDR carriage isolates, 13 serotypes (22.4%) were covered by PCV7, while 11 serotypes (32%) were covered by PCV13. The 6C serotype was the most common multidrug resistant ($n = 10$, 17.2%).

No association between resistance at penicillin, erythromycin, trimethoprim or ceftriaxone and vaccination status was observed (PR = 0.97, 95% CI 0.89–1.05).

4. Discussion

Studies on nasopharyngeal carriage of SP in children have been widely implemented after PCV7 introduction [26–35]. However, only few studies have assessed the epidemiological impact of large scale PCV13 immunization [36].

The impact of PCV13 on SP nasopharyngeal carriage has been recently reported by Cohen et al. who investigated the nasopharyngeal flora of 943 children aged less than 5 years with acute otitis media [36]. On the other hand, data on SP carriage in healthy children are lacking. Given that children affected with a respiratory infection could have a nasopharyngeal flora different than healthy children, epidemiological surveys/updated epidemiological data on nasopharyngeal colonization in healthy children are needed. To our knowledge, this is the first study specifically addressed to describe the SP nasopharyngeal carriage in a population of healthy children aged 3–59 months following the introduction of PCV13 in clinical practice.

The overall prevalence of SP carriage in our study was higher than that reported in pre-vaccine era in Italy (27% vs. 3.5–14.9% respectively) but lower than the last reported by Ansaldi et al. (50%) in Liguria, the Italian region with the highest PCV coverage [16,18,37–40]. This variability is supposed to be dependent on the health status of the studied population (i.e. children with concomitant respiratory tract infections at the time of sampling or healthy), sampling site (nasopharynx vs. oropharynx), seasonality, geography and detection methods (molecular or cultural assays).

The potential role of vaccination on SP nasopharyngeal carriage is a critical issue. In our study the overall prevalence of carriage

Table 4
MIC50 and MIC90 values of *S. pneumoniae* and percentage of susceptibility for all carriage isolates, PCV13 serotypes and non-PCV13 serotypes.

	All serotypes (n: 343)				PCV13 serotypes (n: 75)				Non-PCV13 serotypes (n: 268)				Bp EUCAST	
	MIC50/90	S (%)	I (%)	R (%)	MIC50/90	S (%)	I (%)	R (%)	MIC50/90	S (%)	I (%)	R (%)	S≤	R>
LZD	0.75/1	100.0	0.0	0.0	0.75/1	100.0	0.0	0.0	0.75/1	100.0	0.0	0.0	2	4
ERY	0.094/>256	64.3	0.0	35.7	6/>256	50.0	0.0	50.0	0.094/>256	69.6	0.0	30.4	0.25	0.5
VA	0.38/0.75	100.0	0.0	0.0	0.5/0.75	100.0	0.0	0.0	0.38/0.75	100.0	0.0	0.0	2	2
CHL	3/4	98.3	0.0	1.7	3/4	95.9	0.0	4.1	3/4	99.3	0.0	0.7	8	8
LVX	1/1.5	100.0	0.0	0.0	1/1.5	100.0	0.0	0.0	1/1.5	100.0	0.0	0.0	2	2
CRO	0.032/0.25	96.5	3.2	0.3	0.047/0.5	93.2	5.4	1.4	0.023/0.19	97.4	2.6	0.0	0.5	2
TET	0.19/32	70.9	2.9	26.2	2/24	48.6	4.1	47.3	0.19/24	76.9	2.6	20.5	1	2
SXT	0.125/2	85.3	5.8	8.9	0.125/4	75.7	6.7	17.6	0.125/1.5	87.9	5.5	6.6	1	2
PEN[a]	0.023/0.25	69.2	29.1/0	1.7/30.8	0.047/1.5	60.8	31.1/0	8.1/39.2	0.023/0.19	71.4	27.8/0	0.8/28.6	0.06	2/0.06

Antimicrobial susceptibility (percent susceptibility (*S*), intermediate and resistant (*I* and *R*)) and minimum inhibitory concentration for 50% and 90% of microorganisms. (MIC_{50} and MIC_{90}) values (mg/L). Linezolid (LZD); erythromycin (ERY); vancomycin (VA); chloramphenicol (CHL); levofloxacin (LVX); ceftriaxone (CRO); tetracycline (TET); trimethoprim-sulfamethoxazole (SXT); penicillin G (PEN)

[a] Breakpoints (Bp) non meningitis/meningitis.

Table 5
Antibiotic susceptibility (*N* [%]) to the most common used antibiotics of 343 carriage isolates grouped by vaccine inclusion Antimicrobial susceptibility (percent susceptibility (*S*), intermediate and resistant (*I* and *R*)) and minimum inhibitory concentration for 50% and 90% of microorganisms. (MIC_{50} and MIC_{90}) values (mg/L). For penicillin we used the breakpoints values for "non-meningitis". Using the breakpoints for "meningitis" the value Intermediate will be considerate resistant. Pool G and non-typeable strains not included in the table.

Serotype	*N*	Penicillin[a]				Erythromycin			Trimethoprim/Sulfamethoxazole				Ceftriaxone			
		$MIC_{50/90}$	S	I	R	$MIC_{50/90}$	S	R	$MIC_{50/90}$	S	I	R	$MIC_{50/90}$	S	I	R
PCV7																
6B	6	0.094/0.094	3 (50)	3 (50)	0	0.5/0.75	2 (33.3)	4 (66.7)	3/4	2 (33.3)	0	4 (66.7)	0.047/0.064	6 (100)	0	0
9V	2	0.012/2	1 (50)	1 (50)	0	1/1	1 (50)	1 (50)	0.125/6	1 (50)	0	1 (50)	0.012/0.38	2 (100)	0	0
14	9	0.25/1.5	1 (11.1)	8 (88.9)	0	0.5/1	3 (33.3)	6 (66.7)	0.5/16	6 (66.7)	0	3 (33.3)	0.25/0.75	7 (77.8)	2 (22.2)	0
19F	14	0.19/4	7 (50)	5 (35.7)	2 (14.3)	0.5/1	4 (28.6)	10 (71.4)	0.19/>32	10 (71.4)	1 (7.1)	3 (21.4)	0.125/1.5	12 (85.7)	1 (7.1)	1 (7.1)
23F	7	0.032/0.25	6 (85.7)	1 (14.3)	0	0.75/1	5 (71.4)	2 (28.6)	0.125/2	5 (71.4)	2 (28.6)	0	0.016/0.064	7 (100)	0	0
PCV13																
3	2	0.016/0.032	2 (100)	0	0	0.75/0.75	2 (100)	0	0.125/0.19	2 (100)	0	0	0.008/0.012	2 (100)	0	0
6A	8	0.016/6	6 (75)	1 (12.5)	1 (12.5)	0.75/1	3 (37.5)	5 (62.5)	0.094/0.5	8 (100)	0	0	0.016/0.75	8 (87.5)	1 (12.5)	0
7F	4	0.016/0.023	4 (100)	0	0	0.5/0.75	4 (100)	0	0.064/0.125	4 (100)	0	0	0.012/0.064	4 (100)	0	0
19A	23	0.023/1	17 (73.9)	7 (26.1)	0	0.75/1	10 (43.5)	13 (56.5)	0.125/1.5	20 (87)	2 (8.7)	1 (4.3)	0.047/0.5	23 (100)	0	0
NVs																
6C	32	0.064/0.094	20 (62.5)	12 (37.5)	0	16/>256	13 (40.6)	19 (59.4)	0.125/0.5	31 (96.9)	0	1 (3.1)	0.032/0.064	32 (100)	0	0
7A	4	0.012/0.19	3 (75)	1 (25)	0	0.047/2	3 (75)	1 (25)	0.125/0.5	4 (100)	0	0	0.023/0.032	4 (100)	0	0
9L	1	0.012	1 (100)	0	0	0.094	1 (100)	0	0.19	1 (100)	0	0	0.016	1 (100)	0	0
10A	10	0.016/0.023	10 (100)	0	0	0.064/0.094	10 (100)	0	0.125/0.25	10 (100)	0	0	0.023/0.047	10 (100)	0	0
11A	15	0.016/0.094	13 (86.7)	2 (13.3)	0	0.094/24	12 (80)	3 (20)	0.19/1	14 (93.3)	1 (6.7)	0	0.032/0.064	15 (100)	0	0
11C	2	0.016/0.094	1 (50)	1 (50)	0	0.047/0.064	2 (100)	0	0.064/0.094	2 (100)	0	0	0.047/0.064	2 (100)	0	0
15A	16	0.032/0.38	8 (50)	8 (50)	0	0.125/>256	8 (50)	8 (50)	0.125/0.38	15 (938)	1 (6.2)	0	0.064/0.38	15 (93.8)	1 (6.2)	0
15B	13	0.016/0.125	11 (84.6)	2 (15.4)	0	256/>256	4 (30.8)	9 (69.2)	0.19/2	10 (76.9)	2 (15.4)	1 (7.7)	0.016/0.047	13 (100)	0	0
15C	4	0.032/0.125	2 (50)	2 (50)	0	>256/>256	1 (25)	3 (75)	0.19/0.19	4 (100)	0	0	0.016/0.023	4 (100)	0	0
15F	3	0.012/0.38	3 (100)	0	0	1.5/>256	1 (33.3)	2 (66.7)	1/1.5	1 (33.3)	2 (66.7)	0	0.047/0.25	3 (100)	0	0
16A	8	0.016/0.125	7 (87.5)	1 (12.5)	0	0.094/>256	7 (87.5)	1 (12.5)	0.19/8	6 (75)	1 (12.5)	1 (12.5)	0.023/0.094	8 (100)	0	0
16F	1	1.5	0	1 (100)	0	4	0	1 (100)	0.25	1 (100)	0	0	1	0	1 (100)	0
17A	1	0.38	0	1 (100)	0	>256	0	1 (100)	0.5	1 (100)	0	0	0.38	1 (100)	0	0
18A	1	0.023	1 (100)	0	0	0.064	1 (100)	0	0.19	1 (100)	0	0	0.016	1 (100)	0	0
20	2	0.023/0.064	2 (100)	0	0	0.125/0.125	2 (100)	0	0.19/0.19	2 (100)	0	0	0.012/0.032	2 (100)	0	0
21	13	0.016/0.094	11 (84.6)	2 (5.4)	0	0.094/0.125	13 (100)	0	0.125/0.38	12 (92.3)	1 (7.7)	0	0.016/0.032	13 (100)	0	0
22F	7	0.016/0.094	6 (85.7)	1 (14.3)	0	0.047/0.064	7 (100)	0	0.125/0.19	7 (100)	0	0	0.023/0.047	7 (100)	0	0
23A	29	0.023/0.125	23 (79.3)	6 (23.7)	0	0.094/0.75	26 (89.7)	3 (10.3)	0.125/4	25 (86.2)	1 (3.4)	3 (10.3)	0.016/0.064	29 (100)	0	0
23B	13	0.125/0.25	5 (38.5)	7 (53.8)	1 (7.7)	0.064/0.125	12 (92.3)	1 (7.7)	1/3	8 (61.5)	3 (23.1)	2 (15.4)	0.047/0.064	12 (92.3)	1 (7.7)	0
24A	1	0.023	1 (100)	0	0	0.064	1 (100)	0	>32	0	0	1 (100)	0.016	1 (100)	0	0
24F	3	0.25/1.5	1 (33.3)	2 (66.7)	0	0.19/>256	2 (66.7)	1 (33.3)	24/>32	0	0	3 (100)	0.016/0.125	3 (100)	0	0
25F	1	>32	0	0	1 (100)	256	0	1 (100)	0.125	1 (100)	0	0	1	0	1 (100)	0
29	15	0.032/0.19	13 (86.7)	2 (13.3)		0.094/4	13 (86.7)	2 (13.3)	0.125/0.38	15 (100)	0	0	0.032/0.125	15 (100)	0	0
31	6	0.023/0.064	6 (100)	0	0	0.064/0.094	6 (100)	0	0.125/0.125	6 (100)	0	0	0.012/0.023	6 (100)	0	0
33	6	0.016/0.094	5 (83.3)	1 (16.7)	0	0.094/>256	3 (50)	3 (50)	0.125/0.125	6 (100)	0	0	0.016/0.032	6 (100)	0	0
34	5	0.016/0.047	5 (100)	0	0	0.064/0.094	5 (100)	0	3/4	2 (40)	0	3 (60)	0.032/0.125	5 (100)	0	0
35F	19	0.016/0.125	12 (63.2)	7 (36.8)	0	0.064/0.125	19 (100)	0	0.094/0.19	19 (100)	0	0	0.016/0.19	18 (94.7)	1 (5.3)	0
38	7	0.064/1.5	4 (57.1)	3 (42.9)	0	0.125/4	4 (57.1)	3 (42.9)	0.125/1	7 (100)	0	0	0.064/0.25	7 (100)	0	0
39	3	1/1.5	1 (33.3)	2 (66.7)	0	2/2	1 (33.3)	2 (66.7)	0.19/32	2 (66.7)	0	1 (33.3)	0.25/0.5	3 (100)	0	0
45	1	0.094	0	1 (100)	0	>256	0	1 (100)	0.75	1 (100)	0	0	0.094	1 (100)	0	0

[a] Non meningitis breakpoints *N*, number; NVs, non vaccine serotypes.

was similar in vaccinated and unvaccinated subjects, suggesting that PCV immunization status does not lead to microbial niches for which other pathogen could compete. Moreover, this study shows a reduction of the additional PCV13 serotypes colonization (especially 19A) in children appropriately vaccinated with PCV13, confirming the recent data by Dagan et al. [17]. Considering that the decrease in NP carriage may be predictive of direct protection in vaccinated individuals and indirect effects (herd immunity), large scale PCV13 immunization may have important implications for the control of IPDs [2].

On the other hand we observed a shift in the SP serotype composition, from a mix of vaccine serotypes and non vaccine serotypes (NVS) to almost all NVS both in PCV7 and PCV13 vaccinated children.

In our study serotype 6C was the most frequently isolated regardless the child immunization status. Because of the phenotypically indistinguishable characteristics when using the Quellung reaction, serotype 6C has been differentiated from 6A only recently [41]. Thereafter, a number of reports detailed the prevalence and antimicrobial susceptibility patterns of serotype 6C in invasive disease and nasopharyngeal colonization [41–48]. In USA the prevalence of serotype 6C increased from 1993 to 2009 and it was mainly isolated during bacteremia, meningitis, otitis media or pneumonia [49]. This increased prevalence raised a concern regarding the efficacy of PCV13 immunization against both 6A and 6C. *In vitro* an immunological cross-reactivity between 6A and 6C has been demonstrated. Given the structural similarity of these 2 serotypes, however whether the immunization with PCV13 is protective against serotype 6C disease in humans needs to be clarified [50]. As learned from the rise in 19A invasive diseases despite routine vaccination with PCV7–which contains 19F- the presence of *in vitro* cross-reactivity may not be completely translated into clinical protection [9]. Recently Grant et al. demonstrated that PCV13 elicits functional antibody activity against 6A/B/C and 19A/F [51].

In children with acute otitis media, the biological effect of PCV13 on nasopharyngeal flora has been observed. The documented reduction in 6C carriage rate concurs with serologic data from immunized infants, supporting the hypothesis that the 6A conjugate in PCV13 induces a strong opsonophagocytic response to serotype 6C [36]. Recently, the randomized double-blind trial by Dagan et al. showed that immunization with PCV13 leads to lower acquisition and prevalence of NP colonization than PCV7 for serotypes 6C and 19F and four 4 additional PCV13 serotypes [17].

Our data show a high proportion of 6C SP carriage isolates, regardless of age and vaccination status with trend toward lower values in children appropriately vaccinated for age with PCV13. However, since in our study the number of outcomes was too low to confer adequate power to the comparison.

Interestingly serotype 6C showed the highest MDR prevalence. High resistance to penicillin (non-meningitis breakpoints) was found in 5 (1,7%) SP strains and was mostly associated with serotype 19F. Using breakpoints for meningitis, resistance to penicillin was found in 30.8% of SP strains. Comparing the resistance to penicillin and erythromycin among the SP strains isolated from IPDs in Lombardy (unpublished data) and that of the strains isolated in our study, no differences in MIC_{50} and MIC_{90} values (MIC_S 50% and 90% of the microorganisms, respectively) was found. Overall, strains isolated from carriers were more resistant to penicillin than those isolated from IPD (30.8% and 17% respectively) and equally resistant to erythromycin (35.7% and 32.6% respectively). This higher resistance in SP carriers is not surprising considering that isolates from carriage are frequently more resistant than those isolated from invasive diseases [38,52,53]. In Italy, the observed relatively low level of penicillin resistance in carriage and invasive SP isolates, may reflect a greater accuracy in antibiotic use and treatment policy, and confirm that Streptococcal resistance is directly associated with antibiotic selection pressure on a national level [18,38,52,54].

Our study has some potential limitations. First, we studied a convenience sample of children presenting at their family care pediatricians for routine well care. Thus, our results may not generalize to the whole population of primary care pediatric practices. Second, the age range (3 months–5 years) of the recruited children is relatively large and this might affect the results. However considering that the SP carriage was common in children under 5 years of age we decided not to exclude any age group *a priori*. Third, we used a single-colony method for serotyping; therefore other simultaneously carried serotypes may have been missed. Although molecular methods can detect multiple serotypes, the single-colony method is capable of testing the antibiotic resistance of SP carriage isolates.

Fourth, because of the recent introduction of PCV13 in Italy, only 27.7% of study subject administered PCV13 were appropriately vaccinated for age. A strength of our study is its large sample size. Since the introduction of PCV13 no other studies have examined SP carriage in a such large number of children. This study population provided the unique opportunity to describe the circulation and the antibiotic resistance of colonizing SP.

5. Conclusions

Our study shows a decrease in NP colonization of the additional PCV13 serotypes, in healthy infants and young children following the introduction of PCV13 in routine practice. The greater reduction in prevalence of carriage was observed for serotype 19A. Our data also document a shift toward non vaccine serotypes. Further epidemiological studies of carrier and invasive SP isolates are needed to evaluate changes in the composition of NVS following the large-scale introduction of PCV13. Updated epidemiological data will also guide the development of future vaccines.

Authors' contributions

Dr Zuccotti and Mameli had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Zuccotti, Mameli and Torresani were involved in the study concept and design. Mameli and Daprai were involved in the acquisition of data. Zuccotti, Mameli, Bedogni, Torresani, Daprai and Garlaschi were analyzed and interpreted the data. Zuccotti, Mameli, Bedogni and Daprai were drafted the manuscript. Zuccotti, Gramegna, Faccini, Torresani were involved in critical revision of the manuscript for important intellectual content. Bedogni was done the statistical analysis. Zuccotti, Mameli and Torresani were the study supervisors. All authors have approved the final article.

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