

The impact of private use of PCV7 in 2009 and 2010 on serotypes and antimicrobial resistance of *Streptococcus pneumoniae* carried by young children in Portugal: Comparison with data obtained since 1996 generating a 15-year study prior to PCV13 introduction



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ABSTRACT

In Portugal, the 7-valent pneumococcal conjugate vaccine (PCV7) was not introduced in the national immunization plan but was commercially available between 2001 and 2010. We studied serotype distribution and antibiotic susceptibility of *Streptococcus pneumoniae* carried by children in 2009 and 2010. Vaccination with PCV7 was extracted from children's immunization bulletins and information on recent antimicrobial consumption was obtained through a questionnaire. For comparison, we included data from previous studies conducted since 1996: 1996–1999, 2001–2003, 2006–2007. Pneumococci were isolated from nasopharyngeal samples of 1092 children up to six years old attending day-care in an urban area. Among these, 76% (819/1070) were vaccinated and 62% (677/1092) carried pneumococci. In 2009–2010, serotype replacement was extensive. Carriage of PCV7 serotypes was 4.9% and 5.8%, in 2009 and 2010, respectively, with the majority being of serotype 19F (carried by 4.3% and 4.6% of all participants, respectively). Colonization by serotype 19F was associated with vaccine status (7.7% (19/248) of non-vaccinees vs. 3.5% (29/818) of PCV7-vaccinees, $p=0.010$). Carriage of serotype 19A was high in 2009 and 2010 (8.6% of all participants) consistent with values already observed in 2007; carriage of serotype 6A was <1% (10/1092), indicating a major decline after 2007 (5.8% or 31/538, $p<0.001$). Non-vaccine serotypes increased and serotype 6C became the most frequently carried serotype in 2010 (11.2% (54/481)). High-level resistance to penicillin (MIC ≥ 2 mg/L) showed a decreasing trend ($p<0.001$), whereas resistance to both penicillin and erythromycin increased ($p<0.001$) and was detected in 15–20% of all isolates in 2009–2010, most of which were non-vaccine serotypes. Antimicrobial use decreased over time ($p<0.001$). In conclusion, widespread private use of PCV7 has impacted on colonization leading to near elimination of all PCV7 serotypes except for serotype 19F. Antimicrobial consumption declined but it may be too soon to observe generalized changes in antimicrobial resistance rates.

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1. Introduction

Streptococcus pneumoniae (pneumococcus) often colonizes asymptotically the nasopharynx of young children. However, the pneumococcus can cross biological barriers to cause several diseases such as otitis media, pneumonia, bacteremia and meningitis [1]. Children attending day-care centers (DCC) are considered to be the major reservoirs of pneumococci and cross-transmission in these settings can be extremely high [2].

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Although over 95 pneumococcal serotypes have been described to date, only a few are responsible for the majority of disease [3]. In 2000, a seven-valent pneumococcal conjugate vaccine (PCV7), targeting serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, became available in the USA and soon after in several other countries [4]. More recently, 10-valent (targeting PCV7 serotypes plus serotypes 1, 5, and 7F) and 13-valent (targeting PCV7 serotypes plus serotypes 1, 3, 5, 6A, 7F, and 19A) PCVs have also become available (PCV13 replaced PCV7, both commercialized by the same manufacturer). PCVs not only impact on invasive disease but they also reduce colonization by vaccine serotypes [5–7].

In Portugal, PCVs were not introduced in the National Immunization Plan (NIP) until 2015 but were commercially available with no reimbursement by the state for many years. Specifically, PCV7 became available in June 2001 and was replaced by PCV13 in January 2010. PCV10 became available in April 2009. PCV13 was introduced in the NIP in August 2015. Prior to introduction of PCV in the NIP, the Portuguese Pediatric Society had recommended vaccination of young children with 4 doses of PCV7, starting vaccination at the second month of life [8]. The estimated usage of these vaccines, based on national sales and yearly birth cohorts, is widespread. In particular, among children <1 year, these estimates indicate a gradual increase in PCV7 usage since 2001, reaching 56% in 2003 and 79% in 2007. In 2008 and 2009, PCV7 coverage estimates were 75% and 62%, respectively. PCV10 coverage estimates for 2009 and 2010 were 13% and 7%, respectively. PCV13 coverage estimate for 2010 was 58% (data from IMS and INE/National Statistics Institute).

Since 1996 that pneumococcal carriage studies among children attending day-care in the region of Lisbon have been regularly conducted [9–11]. We have previously shown that by 2006–2007, PCV7 use had led to serotype replacement in colonization [11,12] and that no significant changes on antimicrobial resistance rates had occurred [11,12]. The latter phenomenon was mainly due to the expansion of antimicrobial resistant lineages expressing non-vaccine serotypes [11].

In this study we report serotype distribution and antimicrobial resistance rates in 2009 and 2010, at a time when vaccine coverage among the birth cohort was sustained at 75–80% for nearly 5 years. We also describe for the first time the serotype distribution for all isolates obtained since 1996 expanding on previous studies that focused only on serotypes associated with antimicrobial resistance or specific serotypes [9–15]. Together, the data generated a comparable 15-year-long series, covering the pre- and PCV7-era, allowing the study of the impact of PCV7, antimicrobial use, and secular trends as drivers of changes occurring overtime.

2. Methods

2.1. Ethics statement

The study was registered and approved at the Health Care Center of Oeiras that reports to Administração Regional de Saúde (ARS, “Regional Health Administration”) of Lisboa e Vale do Tejo from the Ministry of Health. The directors of day-care centers approved the study. Written signed informed consent was obtained from parents or guardians of participating children. A numeric code was attributed to each sample upon collection and processed anonymously thereafter.

2.2. Study design

A common study design has been used since 1996. Briefly, the studies were cross-sectional and were carried out during the winter months of January to March of each year (1996–1999, 2001–2003,

2006–2007, 2009–2010), in the Lisbon district (an urban area). Day-care centers covering different socio-economic strata (to reflect the diversity within the population) were invited to participate in the study. Nasopharyngeal samples of children up to 6 years old attending day-care were obtained (one per child) by a trained nurse, essentially following WHO recommendations [16]. A flexible swab (BBL Culture Swab: Becton-Dickinson, Sparks, MD) was inserted through one nostril, parallel to the base of the nasopharyngeal passage until reaching the nasopharynx, turned and removed. If resistance occurred, the swab was gently deviated to enable reaching the base of the nasopharyngeal passage. The swabs were transported to the laboratory at ambient temperature in Stuart medium and plated within four hours of collection. A questionnaire was filled in by the child's parents or guardian with information regarding demographic data and antibiotic consumption. Information on vaccination with PCVs was extracted from the child's immunization bulletin.

2.3. Isolation and identification of pneumococci

Samples were plated onto tryptic soy agar supplemented with 5% sheep blood and 5 µg/mL of gentamicin (one plate per sample). Plates were incubated overnight in anaerobic jars at 37 °C. Pneumococci were identified based on colony morphology, occurrence of α -hemolysis and optochin susceptibility. Bile solubility test was performed to optochin resistant isolates. Suspected colonies, one of each morphology, were picked and streaked onto tryptic soy agar plates. In most samples only one colony morphology was detected by naked eye. Pure cultures were frozen at –80 °C in Mueller–Hinton broth containing 15% (v/v) of glycerol. For this study, only the pure culture of the most abundant colony morphology was characterized as described below and included in the analysis.

2.4. Capsular typing

Capsular type was determined by sequential multiplex PCR using primers previously described ([17] and <http://www.cdc.gov/streplab/pcr.html>). Six group reactions were assigned to detect serotypes 1, 3, 5, 7A/F, 9N/L, 11A, 14, 15A, 15B/C, 16F, 17F, 19A, 19F, 21, 22F, 24A/F, 31, 34, 33A/33F/37, 35F, 38 and serogroups 6, 18 and 23. Primers for the *cpsA* gene were included in each reaction as positive control. Whenever the results were negative or incomplete (at the serogroup level), the Quellung reaction was performed using specific antisera (Statens Serum Institute, Copenhagen, Denmark) [18]. Suspected non-capsulated pneumococci (NT, non-typeable) were identified using a multiplex PCR-based strategy previously described [19].

2.5. Antimicrobial susceptibility testing

Susceptibility to erythromycin, clindamycin, tetracycline, chloramphenicol and sulfamethoxazole-trimethoprim (SXT) was tested by the disk diffusion method (Oxoid, Hampshire, England) according to recommendations and interpretative criteria of the Clinical Laboratory Standards Institute (CLSI) [20]. Minimum inhibitory concentrations (MIC) to penicillin, ceftriaxone and amoxicillin were determined by Etest (Biomérieux, Marcy-l'Etoile, France) and interpreted according to the CLSI guidelines [20]. The breakpoints used for penicillin (oral penicillin V) were ≤ 0.06 , 0.12–1 and ≥ 2 (mg/L) for susceptible, intermediately resistant and resistant, respectively. Corresponding values for amoxicillin and ceftriaxone (both non-meningitis) were ≤ 2 , 4, ≥ 8 and ≤ 1 , 2, ≥ 4 (mg/L), respectively. Multidrug resistance (MDR) was defined as resistance to three or more classes of antibiotics.

2.6. Statistical analysis

The Pearson's Chi-squared test was used to compare differences in the prevalence of serotypes. Significance of temporal trends was tested with the Chi-squared test for trends in proportions; 95% confidence interval for proportions was estimated using the Wilson score-test based method. A p -value of <0.05 was considered statistically significant. The analysis was carried out in R [21].

3. Results

3.1. Population characteristics

In 2009 and 2010, 611 and 481 children were sampled, respectively (Table 1). The age distribution, mean age and gender distribution were comparable between these two years and with previous years. Antimicrobial use, among participants, at sampling or in the month preceding sampling showed a decreasing trend over the years ($p < 0.001$). This decreasing trend was observed for the use of β -lactams and macrolides in the month preceding sampling, but not for the use of β -lactams in combination with a β -lactamase inhibitor (Fig. S1). The proportion of PCV7-vaccinated children (0–6 years-old) with at least one dose was high in 2009 and 2010 (78.3% and 74.2%, respectively), consistent with the high use already observed in 2007 (71.5%). In 2009 and 2010, 87.3% and 89.8% of the children were age appropriately vaccinated and most had received 3 or 4 doses (85.6% in 2009 and 90.5% in 2010). Among children younger than 2 years of age, PCV7 use was even higher: 77.8% in 2009 and 82.6% in 2010. Use of PCV10 was low as only 9 children (2.0%) in 2010 were vaccinated with it; these children were not included in the analysis. None of the children was vaccinated with PCV13. Pneumococcal carriage remained stable in 2009 and 2010 (60.7% and 63.6%, respectively).

3.2. Serotype distribution

In 2009 and 2010, the number of serotypes detected was 29 and 27, respectively. Since 1996, this number ranged between 42 in 2002 and 27 in 2010 (Table 1). In 2009 and 2010, carriage of PCV7 serotypes was 4.9% and 5.8%, respectively (Fig. 1). The most prevalent PCV7 serotype was serotype 19F (carried by 4.3% and 4.6% of all participants, in 2009 and 2010, respectively – Fig. 3). Distribution of serotype 19F was significantly associated with the vaccine status ($p = 0.012$) being identified in 7.5% of non-vaccinated children (19 out of 252) and 3.5% of children vaccinated with at least one dose of PCV7 (29 out of 818). Carriage of other PCV7 serotypes was either not detected or very low, i.e., $\leq 1\%$.

Carriage of the six additional serotypes included in PCV13 (1, 3, 5, 6A, 7F and 19A) was 20.1% and 11.2% in 2009 and 2010, respectively (Fig. 1). Serotype 19A was the most prevalent (8.8% and 8.3% in 2009 and 2010, respectively), followed by serotype 3 (9.3% and 2.3% in 2009 and 2010, respectively). Serotypes 1, 5, 6A and 7F, were either absent or had a very low prevalence ($<2.0\%$).

Carriage of PCV13-only serotypes was higher between 2006 and 2009 (range 16.2–20.9%) than in any other study period. Of interest, carriage of serotype 6A had a major decline after 2007 (from 5.8% to $<2.0\%$, $p < 0.001$); carriage of serotype 3 peaked in 2009 only, being the most frequent serotype among all participants in that year (9.3%); and carriage of serotype 19A increased until 2007 and remained high in 2009 and 2010 (ranking either first or second among all serotypes).

Carriage of serotypes not included in PCV13 was 35.7% and 46.6% in 2009 and 2010, respectively. After 2001, carriage of non-PCV13 serotypes increased consistently, being highest in 2010 (46.6%, Fig. 1). Among this group of serotypes, the most frequently carried

in 2009 and 2010, were serotypes 6C, 15A, 15B/C, 16F, 21, 23A, 23B and non-typeable pneumococci (NT) (Fig. 3). In particular, in 2010, carriage of serotypes 6C (11.2%), 15A (4.8%), 16F (4.4%), 21 (4.8%) and 23B (5.0%) was higher than in any other year (Fig. 3). Serotype 6C carriage stood out as the only non-PCV13 serotype that consistently increased in carriage since 2001 (from 0.4%) becoming the most frequent serotype in 2010 (11.2%).

3.3. Antimicrobial susceptibility

Antimicrobial non-susceptibility rates are shown in Fig. 2 and Table S1. In 2009 and 2010, resistance to penicillin was 1.9% and 2.6%, whereas intermediate resistance to penicillin was 21.6% and 17.0%, respectively (Fig. 2A and B; Table S1). Resistance to erythromycin was higher, i.e. around 25% in the same period (Fig. 2C and Table S1). Dual non-susceptibility to both penicillin and erythromycin was 20.2% and 15.7%, in 2009 and 2010, respectively (Fig. 2D and Table S1).

Comparison with previous years suggested no obvious sustained changes in the prevalence of resistance to penicillin or macrolides, when analyzed independently. However, dual non-susceptibility to these antimicrobials increased significantly after 2003 ($p = 0.024$). Serotypes associated with resistance in 2009 and 2010 were mostly serotypes 19F (included in PCV7 and PCV13), serotype 19A (included in PCV13), and the non-PCV13 serotypes 6C, 15A and NT isolates (Fig. 3).

Regarding other antimicrobial agents, in 2009 and 2010, the highest rates of non-susceptibility were observed for clindamycin and tetracycline (c.a. 19–24%). Non-susceptibility to chloramphenicol, ceftriaxone and amoxicillin were lower, ranging between 1.3% and 8.5%. Since 1996, non-susceptibility to SXT and chloramphenicol showed a significant declining trend ($p < 0.001$ for both), whereas an increasing trend was observed for non-susceptibility to clindamycin ($p < 0.001$). Prevalence of multidrug resistance (defined as resistance to three or more classes of antibiotics among those tested) was 25.9% and 21.6%, in 2009 and 2010, respectively, with no significant trend when compared to data obtained since 1996.

4. Discussion

We studied the long-term impact of PCV7 in carriage, in Portugal, a country with a unique scenario, where PCV7 was only available in the private market but still had a widespread and high usage (reaching 75–80% in 2006–2008, data from IMS and INE/National Statistics Institute). In the present study, we observed an even higher uptake of PCV7.

Comparison of the data obtained in 2009 and 2010 with similar studies conducted since 1996 enabled a 15-year long overview on the evolution of pneumococci, vaccine and antibiotic use, encompassing several years of pre-PCV era and the entire PCV7 era.

One of the most noteworthy results from our study is that, despite having PCV7 exclusively in the private market without any reimbursement scheme in place, significant serotype replacement occurred and, by 2009–2010, all PCV7 serotypes except 19F had become rare. This serotype replacement effect is consistent with what was reported in other countries where PCV7 was introduced in the NIP [22–25]. Serotype 19F, although prevalent, was consistently less frequently carried between 2006 and 2010 when compared to previous years, indicating an impact, even if limited, of PCV7. Of relevance, carriage of serotype 19F was significantly associated with non-vaccinated children. High carriage of serotype 19F was also observed among children from the central region of Portugal and in South Korea after optional use of PCV7 [26,27]. These results suggest that, for this particular serotype, higher

Table 1
Characteristics of the population.

Characteristic	Year ^a (%)										
	1996 <i>n</i> = 584	1997 <i>n</i> = 745	1998 <i>n</i> = 780	1999 <i>n</i> = 937	2001 <i>n</i> = 715	2002 <i>n</i> = 834	2003 <i>n</i> = 765	2006 <i>n</i> = 570	2007 <i>n</i> = 538	2009 <i>n</i> = 611	2010 <i>n</i> = 472
No. of day-care centers	7	12	12	15	10	13	13	11	11	9	6
Mean age (years)	3.6	3.5	3.5	3.3	3.5	3.5	3.5	3.5	3.5	3.3	3.3
Age ^b											
0–<2 years	66 (11.3)	94 (12.6)	111 (14.2)	152 (16.3)	90 (12.6)	98 (11.8)	94 (12.3)	66 (11.6)	78 (14.5)	100 (16.4)	65 (13.8)
2–<4 years	186 (31.8)	218 (29.3)	240 (30.8)	326 (34.9)	217 (30.3)	271 (32.5)	247 (32.3)	184 (32.3)	147 (27.3)	213 (34.9)	180 (38.1)
4–6 years	332 (56.8)	433 (58.1)	429 (55.0)	455 (48.8)	408 (57.1)	465 (55.8)	424 (55.4)	320 (56.1)	313 (58.2)	298 (48.8)	227 (48.1)
Males	284 (48.6)	373 (50.1)	367 (47.1)	459 (49.0)	363 (50.9)	452 (54.2)	387 (50.6)	292 (51.2)	277 (51.5)	325 (53.2)	233 (49.7) ^c
Antibiotic use, %											
At sampling ^d	8.3	7.0	8.2	7.0	5.8	7.0	4.8	4.1	7.0	3.2	4.9
(95% CI)	(6.3–10.8)	(5.4–9.1)	(6.4–10.3)	(5.6–8.9)	(4.3–7.9)	(5.2–9.3)	(3.4–6.8)	(2.8–6.1)	(5.1–9.5)	(2.0–4.9)	(3.2–7.1)
Previous month ^e	26.6	29.0	22.5	23.8	23.5	23.0	16.4	19.3	19.8	19.4	13.6
(95% CI)	(23.1–30.4)	(25.8–32.4)	(19.7–25.6)	(21.1–26.7)	(20.3–27.0)	(19.7–26.6)	(13.6–19.6)	(16.2–22.9)	(16.6–23.5)	(16.4–22.9)	(10.7–16.9)
PCV7 use (≥1 dose)											
0–6 years ^f	–	–	–	–	–	10.7	21.6	57.2	71.5	78.3	74.5
(95% CI)						(8.7–12.9)	(18.8–24.6)	(53.1–61.2)	(67.2–75.3)	(74.8–81.4)	(70.0–77.9)
0–<2 years ^g	–	–	–	–	–	27.6	37.6	78.8	84.7	77.8	79.7
(95% CI)						(19.7–37.1)	(28.5–47.8)	(67.5–86.9)	(74.7–91.2)	(68.6–84.8)	(68.3–87.8)
Pneumococcal carriage, %	46.9	46.8	58.1	62.9	65.6	66.9	72.4	67.9	61.7	60.7	63.8
(95% CI)	(42.9–50.9)	(43.3–50.4)	(54.6–61.5)	(59.7–65.8)	(62.0–68.9)	(63.6–70.0)	(69.1–75.5)	(63.9–71.6)	(57.5–65.7)	(56.7–64.5)	(59.2–67.8)
No. serotypes detected	31	30	35	38	31	42	37	34	29	29	27

Children that had been immunized with PCV10 in 2010 (*n* = 9) were not included in the analysis.

^a Data from 1996 to 2007 are included for comparison.

^b Data missing for 4 children in 1999.

^c Missing gender data for 3 children.

^d Missing data for 3, 20, 9, 29, 80, 245, 163, 15, 12, 8 and 20 children in each year, respectively.

^e Missing data for 39, 28, 26, 50, 102, 268, 184, 47, 28, 40 and 30 children in each year, respectively.

^f Missing data for 1, 65, 16 and 5 children in 2003, 2007, 2009 and 2010, respectively.

^g Missing data for 1, 1 and 1 children in 2003, 2009 and 2010, respectively.

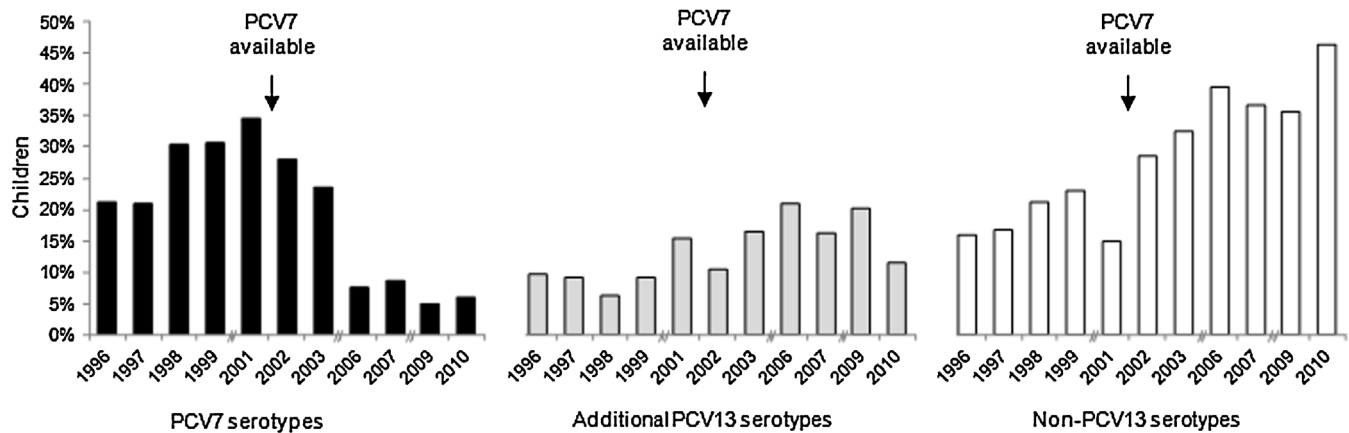


Fig. 1. Carriage of pneumococcal serotypes over time.

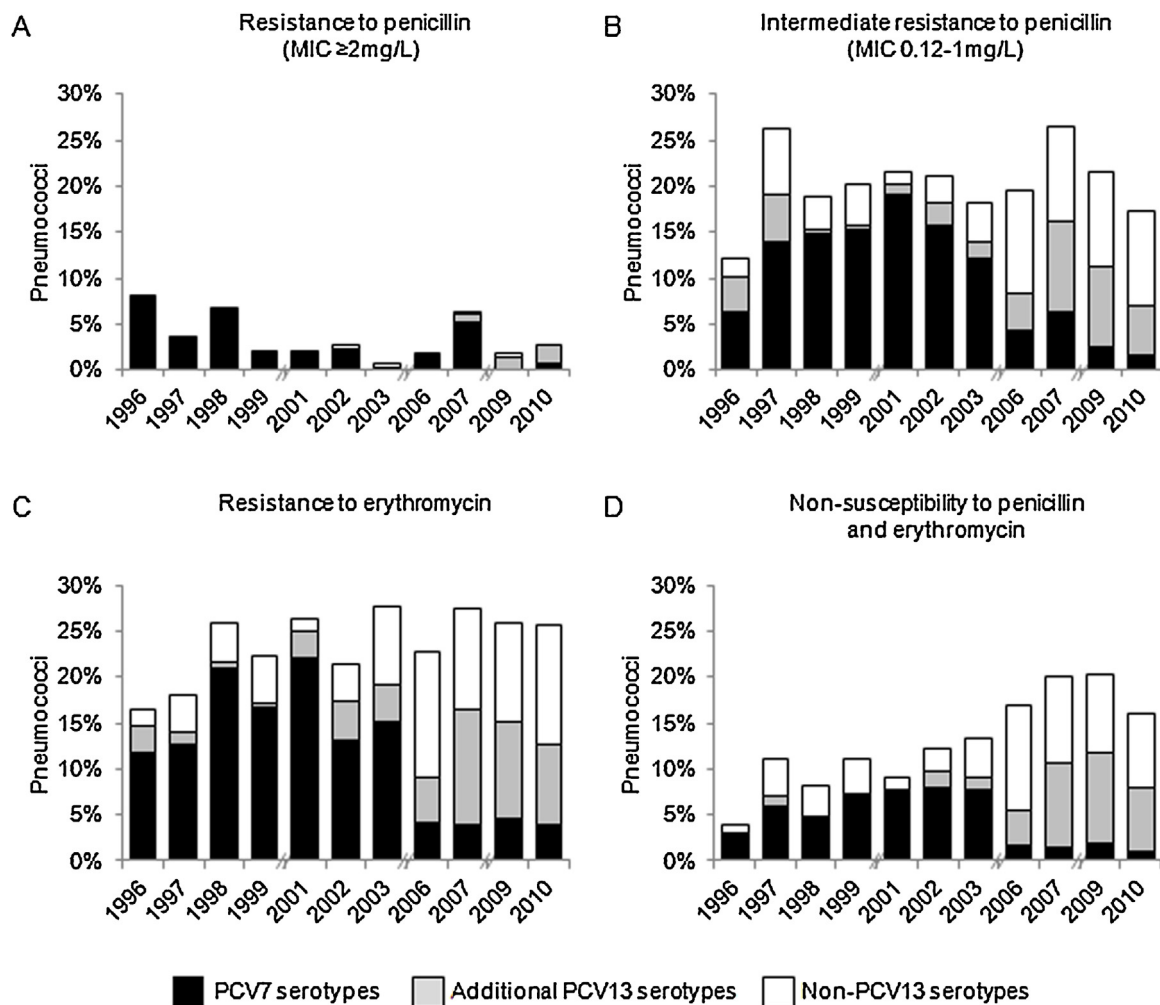


Fig. 2. Pneumococcal non-susceptibility to penicillin and erythromycin. (A) Resistance to penicillin. (B) Intermediate resistance to penicillin. (C) Resistance to erythromycin. (D) Non-susceptibility to penicillin and erythromycin. Data from 1996 to 2007 are included for comparison.

vaccine coverage may be needed to achieve a sustainable low carriage among the population, particularly in those that are not vaccinated.

Analysis of the serotypes included in PCV13 only, a vaccine not yet available at the time of this study, showed that carriage of serotype 19A increased sharply in 2007 and remained one of the most frequently carried serotypes in 2009 and 2010. A similar

increase in the prevalence of serotype 19A was observed in other countries after PCV7 introduction, in carriage isolates and, more importantly, in clinical isolates [6,28]. In Portugal, pediatric invasive pneumococcal disease caused by serotype 19A also increased after PCV7 introduction but declined just before PCV13 introduction, a scenario not observed in our carriage study [29,30]. Recent studies from France on the early impact of PCV13 reported a

reduction in serotype 19A carriage, suggesting a direct impact of the vaccine on this serotype [31,32]. Also, in the USA, Kaplan and co-workers documented a decrease of 58% in the prevalence of serotype 19A among invasive isolates one year after introduction of PCV13 [32].

In our study, carriage of serotype 3 peaked in 2009 probably due to secular trends. For serotype 6A we observed a significant decline in 2009 and 2010 in its carriage. It is unclear whether this resulted

from secular trends, late cross-protection due to PCV7 or both [33].

There is an obvious interest in the identity and characteristics of non-vaccine serotypes increasing significantly following introduction of PCVs. In particular, it is important to identify those serotypes, their virulence potential and their antimicrobial resistance pattern. In our study, serotypes 6C, 15A, 15B/C, 16F, 21, 23B and NT were among the most frequently carried in 2009 and 2010. Of interest, a

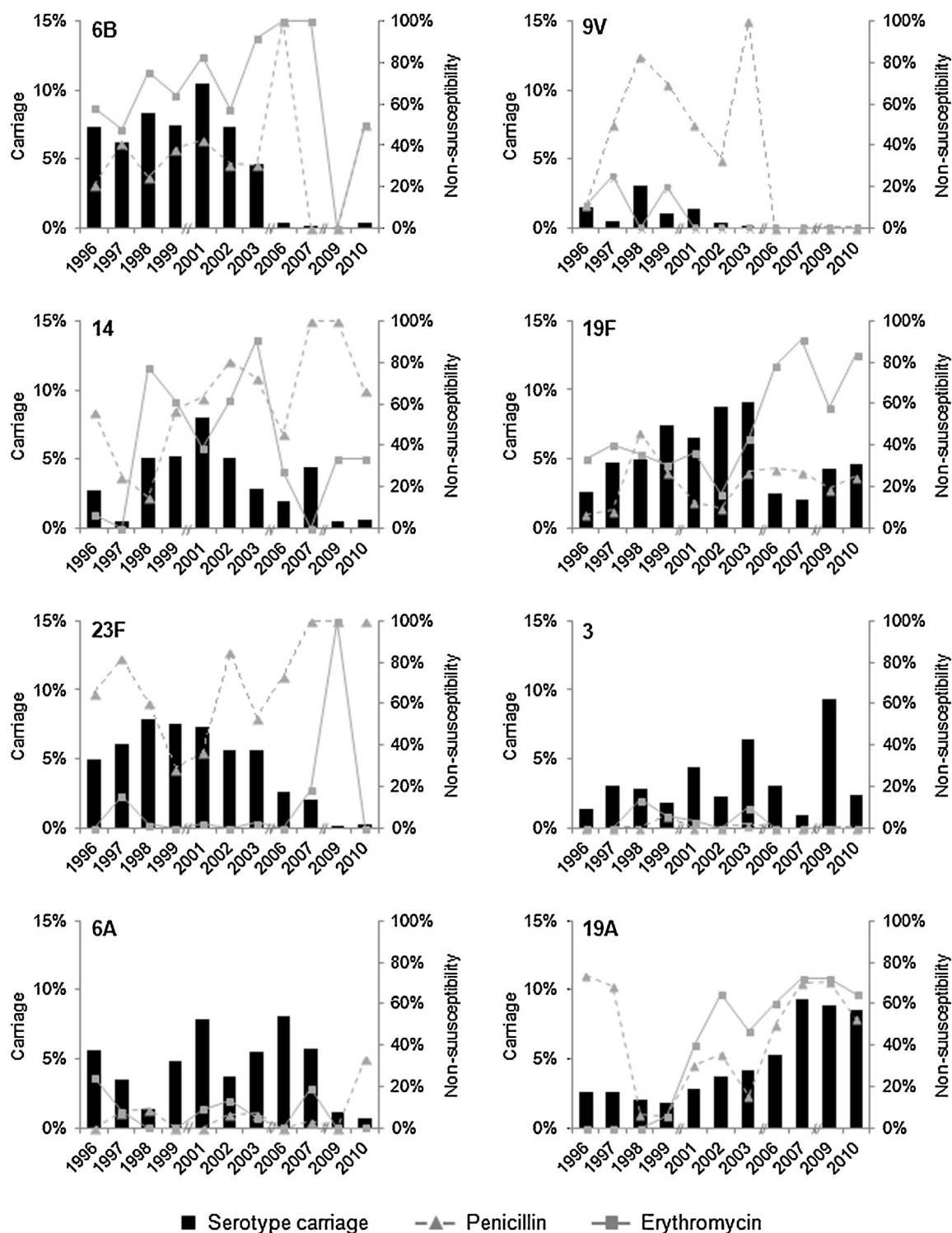


Fig. 3. Carriage of the most frequent serotypes and proportion of isolates non-susceptible to penicillin and/or erythromycin. PCV13 serotypes 1, 4, 5, 7F, and 18C were infrequent and are not shown. Non-PCV13 serotypes with carriage $\geq 3\%$ in 2009 or 2010 are shown. Others: serotypes 7A, 7B/C, 8, 9A, 9L, 9N, 11A, 11C, 11F, 12A, 12B, 13, 16F, 17F, 18A, 18B, 18F, 19C, 20, 22F, 24B, 24F, 25A, 26F, 27, 29, 31, 33A, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 42, 47A.

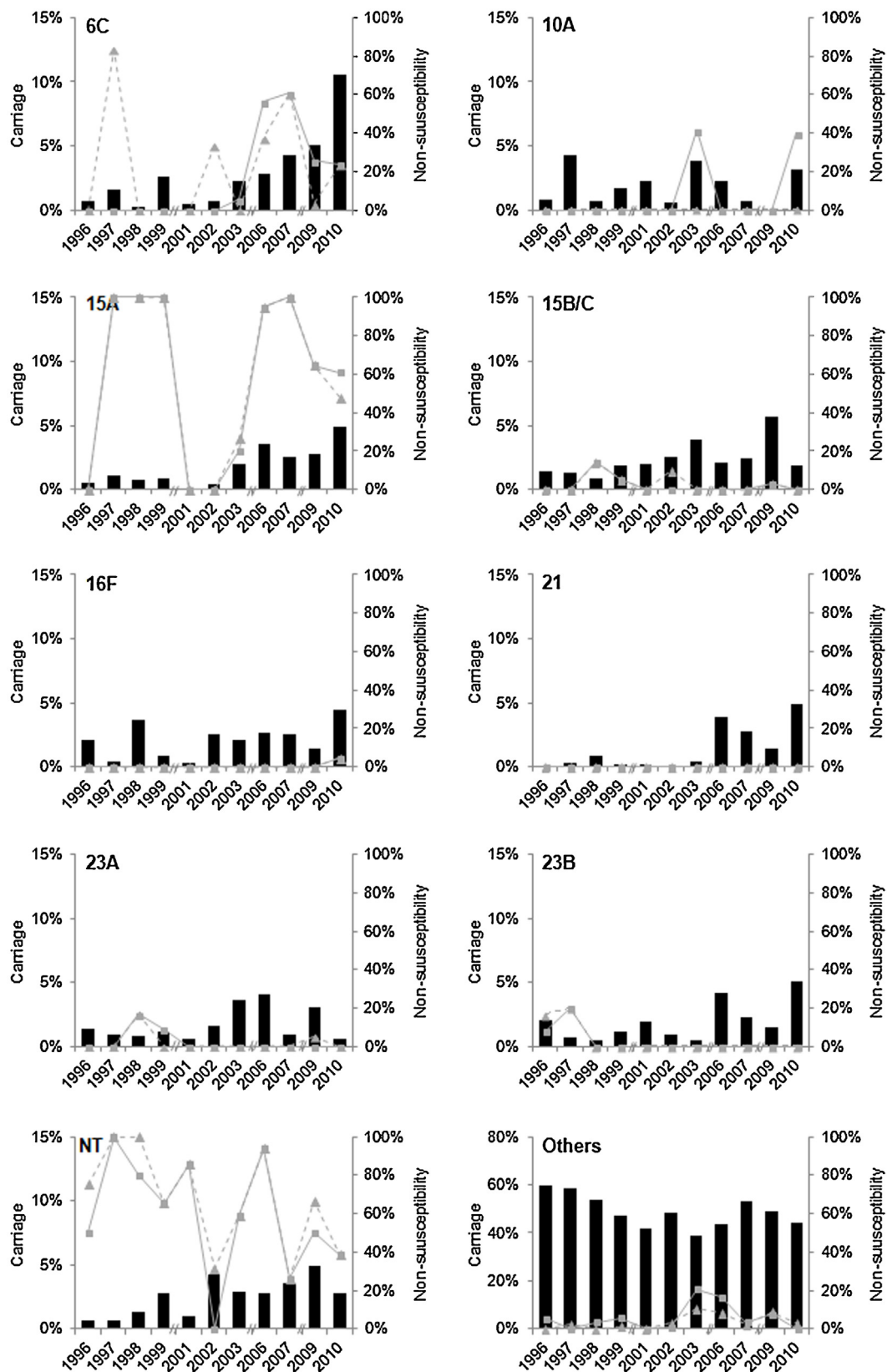


Fig. 3. (Continued)

gradual increase in the carriage of serotype 6C has been observed since 2001, reaching in 2010 the highest proportion of carriage ever observed in our studies for a single serotype. An increase in the prevalence of serotype 6C was reported in other studies as well

during the PCV7 era [34]. Recent studies seem to suggest that PCV13 may impact on serotype 6C due to cross-protection [32].

Antimicrobial resistance remains a main concern. In our study three main observations are of note regarding this topic: (i)

high-level resistance to penicillin, which remains below 5%, is no longer associated with PCV7 serotypes; (ii) dual non-susceptibility to penicillin and erythromycin has been increasing; (iii) since 2006, antimicrobial resistance is mainly associated to serotypes not in PCV7, specifically 6C, 15A, 19A, and NT, whose prevalence increased after PCV7 introduction. Similarly, antimicrobial resistance among clinical isolates in Portugal has also been maintained in recent years [29].

Of relevance, antimicrobial consumption in the population studied declined significantly overtime. This observation is in agreement with data from the European Surveillance of Antimicrobial Consumption Network that indicated a substantial improvement in the quality of antibiotic use in Portugal from 2004 to 2009 when compared to other European countries [35]. Nevertheless, antimicrobial consumption in Portugal remains high when compared to Northern European countries [35]. Our data on the evolution of serotypes associated with antimicrobial resistance also suggested that antimicrobial pressure remains high in the population [11].

Our study has some limitations concerning the direct comparison of collections obtained in different years. Although the sampling protocol and the laboratory procedures for pneumococcal isolation were the same between 1996 and 2010, pneumococcal carriage rates observed in 1996 and 1997 were lower than in all other years. The reasons for this are not understood but may have been due to less experience in the nasopharyngeal sampling technique of the nurses at the beginning of the project. However, as the paper focus on serotypes and antibiotic resistance we do not think that this has affected the results, as a lower capacity to detect pneumococci (due to sampling) would affect the entire pneumococcal population in a similar way. Therefore, the relative proportions of serotypes and resistance patterns should not be affected. The second limitation concerns the number of day-care centers and children enrolled in each year that varied over time (between 6 and 15 centers, and 472–937 participants). Due to practical reasons it was not possible to maintain through time a high number of day-care centers and participants in the project. Of note, the day-care centers were all located in the same geographical area and served a common population and therefore the results obtained are unlikely to have been biased. A third potential limitation was the inclusion of children taking antimicrobials at the time of sampling or within the previous month. The decision to include these children was consistent throughout the 15-year period. As antimicrobial use in this population is significant by excluding these children we would be disregarding an important fraction of the population. Of note, we re-analyzed serotype distribution per year and among non-susceptible isolates after excluding all children on recent antimicrobial use (i.e. in the previous month or at sampling) and obtained comparable results (data not shown).

In conclusion, although PCV7 is not included in the NIP in Portugal, its widespread use has impacted on colonization patterns with a sustained effect observed between 2006 and 2010. Prevalence of vaccine types has been significantly reduced, whereas prevalence of non-vaccine types has increased, and in 2009 and 2010 the only PCV7 serotype that remained prevalent was serotype 19F, being associated with non-vaccinated children. Antimicrobial resistance remains a concern suggesting that additional efforts to contain antimicrobial use must be implemented. PCV13 became available in Portugal in 2010 after this study. These results provide a valuable baseline to monitor serotype evolution in the era of PCV13.

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Authorship/contribution

The study was conceived by HL, ABA and RSL. Data acquisition, analysis and interpretation were done by all authors. HL and RSL contributed with reagents and materials. The manuscript was drafted by SF, ACP and RSL and critically revised by all authors. All authors read and approved the final version of the manuscript.

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Conflict of interest: RSL reports grants (outside the submitted work) administered through her university and honoraria for serving on speakers bureaus of Pfizer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2016.02.045>.

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