**Impact of the 13-valent pneumococcal conjugate vaccine on *Streptococcus pneumoniae* multiple serotype carriage**

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**ABSTRACT**

**Introduction:** Pneumococcal multiple serotype carriage is important for evolution of the species and to understand how the pneumococcal population is changing with vaccination. We aimed to determine the impact of the 13-valent pneumococcal conjugate vaccine (PCV13) on multiple serotype carriage.

**Methods and Materials:** Nasopharyngeal samples from fully vaccinated pneumococcal carriers (4 doses of PCV13, n=141, aged 18-72 months) or from non-vaccinated pneumococcal carriers (0 doses of any PCV, n=140, same age group) were analyzed. Multiple serotype carriage was evaluated by DNA hybridization with a molecular serotyping microarray that detects all known serotypes.

**Results:** Vaccinated children had a lower prevalence of multiple serotype carriage than the non-vaccinated group (20.6% vs 29.3%, p=0.097), and a significantly lower proportion of PCV13 serotypes (6.4% vs 38.5%, p=0.0001). PCV13 serotypes found among vaccinated children were mostly detected as a minor serotype in co-colonization with a more abundant non-vaccine serotype. Vaccinated children were colonized by a significantly higher proportion of commensal non-pneumococcal *Streptococcus spp*. (58.2% vs 42.8%, p=0.012). In vaccinated children there were significantly less non-vaccine type (NVT) co-colonization events than expected based on the distribution of these serotypes in non-vaccinated children.

**Conclusions:** The results suggest that vaccinated children have lower pneumococcal multiple serotype carriage prevalence due to higher competitive abilities of non-vaccine serotypes expanding after PCV13 use. This might represent an additional benefit of PCV13, as decreased co-colonization rates translate into decreased opportunities for horizontal gene transfer and might have implications for the evolution and virulence of pneumococci.

**KEY WORDS:** *Streptococcus pneumoniae*;multiple serotype carriage; co-colonization; pneumococcal conjugate vaccine; PCV13; microarray.

**INTRODUCTION**

*Streptococcus pneumoniae* is an important cause of infectious disease, with a high rate of mortality worldwide, particularly among young children, the elderly and the immunocompromised [1].

Despite the high burden, invasive pneumococcal disease is incidental [2, 3 ]. Nasopharyngeal colonization is the natural lifestyle for the pneumococcus, with particularly high prevalence among young children [4]. Colonization is key to pneumococcal biology as it precedes disease, facilitates transmission between hosts for perpetuation of the species, and allows sustained evolution of the species to take place.

The pneumococcus evolves mainly by recombination through horizontal gene transfer occurring when multiple strains or serotypes of pneumococci coexist, a phenomenon also known as co-colonization. Likewise, horizontal gene transfer with closely related co-colonizing commensals such as *S. mitis* and *S. pseudopneumoniae* can also occur [5].

Co-colonization or multiple serotype carriage is frequent. Recent studies reported multiple serotype carriage prevalence of up to 40% in children [6-8]. Accurate detection of multiple serotype carriage in surveillance studies is relevant (i) for the understanding of intra-species interactions, (ii) to obtain a comprehensive knowledge of how the pneumococcal population is being altered by anti-pneumococcal vaccination, and (iii) to predict the vaccine impact on disease when using models based on carriage prevalence [9-11].

In recent years, with the increasing availability of highly sensitive serotyping methods that are able to detect multiple serotype carriage, reports on co-colonization are becoming more frequent [6-8, 12, 13]. In a previous study conducted to determine the prevalence of multiple serotype carriage among Portuguese children and the impact of vaccination with the 7-valent pneumococcal conjugate vaccine (PCV7), we demonstrated that PCV7 vaccinated children were significantly less co-colonized than non-vaccinated children due to an uneven distribution of serotypes selected by PCV7 in single and co-colonization events [8].

The 13-valent pneumococcal conjugate vaccine (PCV13) became commercially available in Portugal in January 2010 replacing PCV7. Although at the time of this study none were introduced in the National Immunization Program (NIP), these vaccines are highly prescribed and their usage has been recommended by the Portuguese Pediatric Society [14]. For this reason, vaccine coverage has been high, reaching 63% by 2012 (data from the National Statistics Institute and IMS HealthTM). Very recently, in June 2015, PCV13 was introduced in the NIP on a scheme of two doses followed by a booster dose.

The impact of PCV13 on carriage is being studied in several countries and there are already some studies addressing this issue [15-17]. However, to our best knowledge the impact of this vaccine on carriage of multiple serotypes and co-colonization with other *Streptococcus spp.* has not been addressed.

The aims of this study were to determine the impact of PCV13 on pneumococcal multiple serotype carriage and evaluate whether the results were globally comparable to those obtained for PCV7.

**MATERIALS AND METHODS**

**Study design**

Nasopharyngeal (NP) swabs collected from healthy children attending day-care centers in Oeiras and Montemor-o-Novo, Portugal, were retrospectively selected. Samples were collected in the winter months of January to March between 2011 and 2015. A total of 2,607 children were sampled and 1,565 (60.0%) were found to be pneumococcal carriers.

The following criteria were used for selection of samples to be analyzed in this study: (i) swabs were obtained from children aged 18-71 months; (ii) children had not received antibiotic within the month preceding sampling; (iii) children were either non-vaccinated (i.e. had not received any PCV) or were fully PCV13-vaccinated *i.e.*, had received four PCV13 doses; and (iv) swabs yielded a pneumococcal positive culture.

Five hundred and fifty-seven children fulfilled criteria (i), (ii) and (iii): 303 had not been vaccinated with any PCV (of these, 60.1% (n=182) were pneumococcal carriers) and 254 were fully PCV13-vaccinated (of these 66.5% (n=169) were carriers). For this study, 300 samples from the non-vaccinated and fully vaccinated groups (n=150 for each group) were randomly selected from pneumococcal carriers to meet criterion (iv). The 300 samples were analyzed by the microarray as described below.

**Ethics statement**

This study was approved by the Ethics Research Committee of the NOVA Medical School/Faculdade de Ciências Médicas – Universidade Nova de Lisboa (CEFCM) (47/2014/CEFCM). Samples were collected upon signed informed consent from the parents or guardians of participating children. All information was processed anonymously.

**Sample collection and isolation of pneumococci**

NP samples were collected by pediatric nurses. In 2011 and 2012 mini-tip calcium alginate sterile swabs were used and inoculated directly within 4 hours on a primary selective plate of 5% blood trypticase soy agar containing gentamicin (5 mg/liter) to select for *S. pneumoniae*. Samples from 2014 and 2015 were collected and isolated according to the standard procedures recommended by the WHO [18]. Swabs, the total bacterial lawn of the primary gentamicin blood plate and pneumococcal isolates were frozen at -80ºC in 1ml of STGG medium containing 30% glycerol.

**DNA isolation**

STGG tubes containing the nasopharyngeal swab were thawed on ice and vortexed. 50 µl aliquots were plated onto tryptic soy blood plates supplemented with gentamicin using a spreader and incubated overnight at 37ºC in 5% CO2. On the following day, a plate sweep of the total bacterial growth was collected and DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Germany).

**Detection of multiple serotype carriage by microarray**

The Senti-SPv1.5 *S. pneumoniae* molecular serotyping microarray (BUGS Bioscience, UK) was used following standard protocols previously described [12, 13]. Genomic DNA ULS labeling and hybridization protocols were used and microarray slides were scanned using a high-resolution microarray scanner (Agilent Technologies, USA). Microarray data was analyzed using a Bayesian hierarchical model to determine the serotype, or combination of serotypes, present in the sample and to assign their relative abundance [19]. For the interpretation of the microarray quantification results, a serotype was classified according to its relative abundance in the sample as: dominant if ≥70%; co-dominant if >30% and <70% and minor if ≤30%.

**Statistical analysis**

Statistical significance was assessed using Fisher’s exact tests. For all analyses differences were considered statistically significant when p<0.05.

Permutation analysis was performed to test if serotypes were found in multiple serotype carriage at frequencies significantly different from those expected by chance alone. Serotype attributions were randomly allocated to children (including those not colonized) 5000 times. Serotype frequencies were maintained. The p-values obtained for all serotypes were corrected for multiple testing by controlling the False Discovery Rate below 0.20 [20].

**RESULTS**

**Molecular serotyping and detection of co-colonization**

The microarray analysis detected *S. pneumoniae* DNA in a total of 281 samples – 140 samples collected from non-vaccinated children and 141 samples collected from vaccinated children. Samples in which the microarray failed to detect pneumococcal DNA were excluded from the study (n=19). In four of these samples one encapsulated pneumococcal strain had been isolated before, but probably due to low abundance and sampling, it was not detected when the DNA of the primary culture was probed in the microarray. In the remaining 15 samples, putative non-encapsulated pneumococci were isolated and were later confirmed to be strains of other closely related *Streptococcus spp.*

Among the 281 samples containing pneumococci, the microarray identified 345 pneumococcal strains of known serotypes, 30 pneumococcal strains of non-encapsulated lineages (herein called non-typeable or NT) and 302 non-pneumococcal *Streptococcus spp*. strains. The pneumococcal population was distributed throughout 40 serotypes and NT strains (Figure 1A). Pneumococcal multiple serotype carriage was detected in 25% of the samples (70/281). Half of the samples (50.5%, 142/281) contained at least one other *Streptococcus spp*. strain in addition to *S. pneumoniae*.

The microarray identified three types of non-typeable non-encapsulated pneumococci that are genetically distinct: NT2, NT3b and NT4b detected in 1.1% (n=3), 7.5% (n=21), and 2.1% (n=6) of the samples, respectively [21, 22]. For subsequent analysis we have grouped non-encapsulated strains into a single group (NT), which was collectively the most prevalent pneumococcal type.

The most abundant *Streptococcus* species (other than pneumococci) identified were *S. mitis*, *S. oralis*, *S. infantis* and *S. salivarius* present in 46.3% (n=130), 34.9% (n=98), 32.7% (n=92) and 29.9% (n=84) of the samples, respectively. Other species identified included *S. pseudopneumoniae*, *S. sanguinis*, *S. constellatus*, *S. anginosus* and *S. intermedius*, present in up to 5% (n=14) of the samples. Among these samples, in some cases the microarray detected the presence of a few capsule biosynthesis gene homologues, but never the full set of *cps* genes expected for any known pneumococcal serotype.

In general, serotypes found in co-colonization had comparable frequencies as minor or non-minor serotypes. The exception to this scenario were NT pneumococci, which were found as minor serotype in the majority of co-colonization events (p=0.0164) (Figure S1). Still, the ranking frequencies of several serotypes would change if only the dominant serotype in the samples had been detected (Figure 1A).

Comparison between the serotype distributions obtained by microarray and by culture showed that the serotypes most frequently detected by culture were the same as those most frequently detected by the microarray (Figure 1). However, due to higher sensitivity of the microarray, the prevalence of individual serotypes was higher with this method compared to culture. Also, the microarray detected a higher number of serotypes (41 detected by microarray vs 32 detected by culture).

**Pneumococcal co-colonization in PCV13 vaccinated and non-vaccinated carriers**

The prevalence of pneumococcal multiple serotype carriage among PCV13 vaccinated carriers was lower (20.6%, 29/141) than among the non-vaccinated group (29.3%, 41/140), although the difference was not statistically significant (p=0.0973). In both groups most multiple serotype carriage samples contained two serotypes and a maximum of four serotypes were detected in 5 samples. In multiple serotype carriage samples, a clear dominance of one serotype was noted in most cases with no significant differences between the two groups (Table 1).

Overall carriage of PCV13 vaccine types was significantly lower in the vaccinated group, compared to the non-vaccinated group (6.4% (9/141) vs 38.5% (57/140), p=0.0001) (Figure 2).

Among the few (n=9) vaccinated children that carried PCV13 serotypes, six had them in multiple carriage events with a non-vaccine type (NVT); in five of these events the vaccine type was in minority. By contrast among the 54 non-vaccinated children that carried PCV13 serotypes, in the majority (37, 69%) it was found as a single serotype. In the remaining carriers, PCV13 serotypes were found in multiple carriage events and were dominant in the majority of the events (11/17) (Figure 3).

Of note, serotype 19F accounted for 40% (26/66) of all PCV13 strains identified by the microarray and was the most prevalent PCV13 serotype in vaccinated and non-vaccinated children, both in single and co-colonization events (Figure 3).

Of all types, NT strains were the most prevalent pneumococcal type identified in single and multiple carriage events, being present in 11.4% (32/281) of co-colonized samples. Apart from type NT3b which was found as a dominant strain in three multiple carriage events, all non-encapsulated strains were found co-colonizing as a minor serotype.

**Serotype distribution**

To investigate if individual serotypes were detected in multiple carriage events at a frequency significantly different from that expected by chance a permutation analysis was performed taking into account the overall serotype distribution in single and multiple carriage events. Serotypes 3 (p=0.036), 22F (p=0.022), 31 (p=0.036), 34 (p=0.032), 35B (p=0.017) and 35F (p=0.040) were detected in multiple carriage events at a frequency significantly lower than expected. No serotypes were found in multiple carriage at a frequency significantly higher than expected by chance (Figure 1).

Comparison of the relative fraction of VTs occurring in single and multiple serotype carriage events among vaccinated and non-vaccinated children revealed no significant differences between the two groups (probably due to low statistical power resulting from low occurrence of VTs in vaccinated children) albeit suggesting that this proportion tended to be higher in vaccinated children: 6 out of 9 (66.7%) VTs occurred in multiple carriage in vaccinated children compared with 19 out of 56 (33.9%) VTs occurring in multiple carriage in non-vaccinated children (p=0.076) (Figure 2).

By contrast, the relative fraction of NVTs occurring in single and co-colonization events was significantly different among vaccinated and non-vaccinated children and the trend was opposite to the one observed for VTs: 62 out of 171 (36.2%) NVTs occurred in multiple carriage in vaccinated children, while for non-vaccinated children, 75 out of 137 (54.7%) NVTs were found in multiple carriage (p=0.0013) (Figure 2).

**Associations in co-colonization**

We investigated associations among the co-colonized samples. As the number of serotypes present in co-colonization was high (n=38), the frequency in which specific pairs of serotypes were found together was low. Vaccinated children were co-colonized by a lower number of serotypes (n=26) compared to the non-vaccinated group (n=35), which translated into a less complex network of pairings (Figure 4).

Among vaccinated co-colonized children the most frequent pneumococcal types were NT strains and serotypes 16F, 11A, 15B/C and 21. Among non-vaccinated co-colonized children the most frequent types were NT, 19F, 23B, 6C, 15B/C, and 16F.

Carriage prevalence of other *Streptococcus* *spp*. was high in both groups but significantly higher in vaccinated children, compared to the non-vaccinated group (58.2% (n=82 out of 141) vs 42.8% (n=60 out of 140), p=0.0122) (Figure 4).

The most frequent association detected was between non-encapsulated pneumococci and non-pneumococcal *Streptococcus spp*. Other frequent associations of *S. pneumoniae* serotypes were 19F-NT, 15B/C-NT, 6C-NT (Figure 4).

**DISCUSSION**

We used a molecular serotyping microarray to evaluate the impact of PCV13 on pneumococcal multiple serotype carriage by comparing a group of children who had received four PCV13 doses with another group that had not been vaccinated with any PCV. Microarray was recently described as the top-performing method currently available to study multi-serotype carriage [23].

Our results show that vaccinated children had a lower prevalence of multiple serotype carriage compared to non-vaccinated children (20.6% vs 29.3%), although this difference was not statistically significant.

As expected, we observed that use of PCV13 in Portugal has led to serotype replacement in vaccinated children, a result that is in line with findings in other countries [24, 25]. The use of the microarray has shown that vaccinated children not only carry fewer VTs but also tend to have them as a minor serotype in multiple carriage with a NVT. On the other hand, among non-vaccinated children carriage of VTs is frequent and often found in single carriage events. When in multiple carriage VTs tend to be the dominant type in this naïve group.

Moreover, we observed that vaccinated children were colonized by a lower number of serotypes and had a higher prevalence of NT pneumococci and other *Streptococcus spp.*, compared to the non-vaccinated group, suggesting niche replacement by less virulent strains. This observation suggests that PCVs, by eliminating highly competitive serotypes from the nasopharyngeal niche, are allowing the expansion of commensal streptococci. This hypothesis is further supported by a study in which the nasopharyngeal microbiota profiles of vaccinated and non-vaccinated children were compared, showing expansion of other *Streptococcus spp.* and other commensals in vaccinated children [26].

Yet another difference observed between vaccinated and non-vaccinated children was the asymmetric distribution of NVTs in single and multiple carriage events when the two groups were compared. In vaccinated children the proportion of multiple carriage events with NVTs was lower than would be expected by chance alone, based on the prevalence of these types in the non-vaccinated group. This observation supports the observation that pneumococcal serotypes have different competitive abilities [8, 27, 28] and that some NVTs that became more prevalent following PCV13 introduction might be highly competitive, preventing their co-existence with other pneumococci. This hypothesis is further supported by two observations: (i) vaccinated children, where serotype replacement was more pronounced, presented lower co-colonization prevalence; and (ii) some prevalent NVTs (22F, 31, 34, 35B and 35F) where found less frequently than expected in multiple serotype carriage events (while no serotypes were found more frequently than expected in multiple serotype carriage events). Of interest, two of these serotypes - 22F and 35B - are becoming highly prevalent in the PCV13 era. This could result in a further decrease in multiple serotype carriage [25, 29].

The results obtained in this study are in agreement with a previous study with a similar aim conducted in the PCV7 era [8]. In both studies we found lower co-colonization rates among vaccinated children and an asymmetric distribution of NVTs in single and multiple carriage events in this group. Results from both studies suggest that PCVs might have the additional benefit of decreasing pneumococcal co-colonization events, and thus, the opportunities for horizontal gene transfer in pneumococci [30]. However, it is also possible that our observations reflect a transient phenomenon of early vaccine effect before equilibrium in serotype distribution has been reached [31]. Further studies on pneumococcal multi-serotype carriage should be able to clarify these hypotheses.

Our study has some limitations. First, there were differences in the methodology for isolation of *S. pneumoniae* in the years of 2011/2012, compared to 2014/2015 that could potentially interfere with results. However when all analyses were repeated excluding samples from 2011 and 2012 the results obtained mimicked the ones reported in this study (apart from the difference in carriage of *Streptococcus spp.* between vaccinated and non-vaccinated children, which became non-significant but still showed the same trend) suggesting that the differences observed between groups were not related to the isolation method. Second, the culturing step of the nasopharyngeal swab could potentially have altered the composition of the sample and, thus, interfere with the detection and relative quantification of pneumococci, or induce a bias towards the detection of viable cells only, resulting in false-negative results. Nevertheless, we are convinced that the increase in the total pneumococcal DNA induced by the culturing step is an advantage that surmounts the referred limitations, as direct analysis of swab DNA results in a lower sensitivity to detect minor serotypes in low abundance. Third, the selection of samples culture positive for *S. pneumoniae* could be regarded as a limitation given the lower sensitivity of culture-based methods in the detection of pneumococcal carriage. This approach may have resulted in the exclusion of unidentified pneumococcal carriers (and hence multiple serotype carriers). On the other hand, this approach enabled us to probe a high number of samples which were certain to contain pneumococci and therefore maximized the likelihood of detecting multiple carriage events.

Our study has also some strengths. To our knowledge, this is the first study focusing on the impact of PCV13 on pneumococcal co-colonization, reporting differences between vaccinated and non-vaccinated children that might represent novel benefits of PCVs. Also, the use of a highly sensitive microarray allowed us to detect minor serotypes that would otherwise be missed, and to assess how the pneumococcal population structure might be altered by the detection of those serotypes, namely by the unmasking of some serotypes that might be more prevalent than initially thought. In addition, because the microarray incorporates all capsule biosynthesis genes, plus reporters for the *S. pneumoniae* genome backbone and species discrimination, the use of this methodology allowed us to correctly identify pneumococcal strains and to report on the prevalence of closely related *Streptococcus spp*.

A word of caution regarding our study is the fact that we cannot exclude the hypothesis that the observed effect of PCV13 on co-colonization might by restricted to the population studied here. Although we have now addressed this effect of PCVs on co-colonization twice with concordant results [8], the observed effects might be a consequence of the serotype distribution in our setting. Additional studies would be of interest to ascertain whether this is the case.

In summary, this study corroborates our previous observations regarding the impact of conjugate vaccines on pneumococcal co-colonization, showing that vaccinated children have lower multiple serotype carriage rates than non-vaccinated children. This observation has implications for the virulence and evolution of pneumococci, as it might result in decreased opportunities for horizontal gene transfer between strains. Of note, we show a very high prevalence of non-encapsulated pneumococci and *Streptococcus spp*., particularly among vaccinated children. The role of these species in the nasopharyngeal ecosystem and in the evolution of pneumococci are questions that remain to be addressed but overall our findings suggest a niche evolution towards commensality.

**Authorship/Contribution**

The study was designed by RSL. Data acquisition, analysis and interpretation were performed by CV, KG, JH, and RSL. The manuscript was drafted by CV and RSL and critically revised by all authors. All authors read and approved the final version of the manuscript.

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**CONFLICTS OF INTEREST**

RSL reports grants administered through her university and honoraria for serving on speakers bureaus of Pfizer. JH reports grants administered through his university from GSK, Sanofi-Pasteur, Pfizer and is co-founder of BUGS Bioscience, a not-for-profit spin-out company. All other authors have no conflicts of interest to declare.

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**TABLE**

|  |  |  |  |
| --- | --- | --- | --- |
| **Characteristic** | **PCV13 vaccinated**  **(total=141)**  **n (%)** | **Non-vaccinated**  **(total=140)**  **n (%)** | **p-valuea** |
|  |  |  |  | |
| **Multiple serotype carriage** | 29 (20.6%) | 41 (29.3%) | 0.0973 | |
|  |  |  |  | |
| **No. serotypes detected**  Two  Three  Four  **Relative quantification**  Dominance of one strain  Co-dominance | 20 (69.0%)  8 (27.6%)  1 (3.4%)  19 (65.5%)  10 (34.5%) | 32 (78.1%)  6 (14.6%)  3 (7.3%)  34 (82.9%)  7 (17.1%) | 0.4182  0.2305  0.6369  0.1560 | |
|  |  |  |  |

**Table 1.** Comparison between nasopharyngeal samples obtained from PCV13 vaccinated and non-vaccinated pneumococcal carriers.

aFisher’s exact test.

**FIGURE LEGENDS**

**Figure 1. Serotype ranking obtained through detection of carriage by the capsular microarray (A) or by culture (B).** Black, prevalence in single carriage, white, prevalence in multiple serotype carriage. Asterisks, frequency in multiple serotype carriage events is significantly lower than expected by chance.

**Figure 2. Single and multiple serotype carriage events by PCV13 vaccine types (VT) and non-PCV13-vaccine types (NVT) in PCV13 vaccinated and non-vaccinated children.** Black, colonization with a single VT,dotted gr**a**y, colonization with a single NVT, white, colonization with two or more VTs, dark gray, colonization with one or more VTs with one or more NVTs, and light gray, colonization with two or more NVTs. Asterisks indicate total prevalence of multiple serotype carriage.

**Figure 3. Carriage and relative abundance of PCV13 serotypes in vaccinated and non-vaccinated children, in single and multiple serotype carriage events.** Bars correspond to pneumococcal samples. Black, PCV13 serotype; white, non-PCV13 serotype (NVT). Multiple carriage events are divided in PCV13-PCV13 and PCV13-NVT, according to types of serotype associations.

**Figure 4. Node plot of strains co-colonizing vaccinated and non-vaccinated children.** The size of each node is proportional to the number of pairings in which each serotype is found. The width of the lines is proportional to the frequency in which two serotypes are found together.

**SUPPORTING INFORMATION**

**Figure S1. Frequency of serotype occurrences as minor or dominant/co-dominant strain in multiple serotype carriage events.** Minor serotype defined as having a relative abundance ≤30% of total pneumococcal DNA; Dominant/co-dominant serotype defined as having a relative abundance >30% of total pneumococcal DNA.