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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.

1. 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].

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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 Health™). 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.

2. Materials and methods

2.1. 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 2607 children were sampled and 1565 (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.

2.2. 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.

2.3. 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 h on a primary selective plate of 5% blood trypticase soy agar containing gentamicin (5 mg/l) to select for *S. pneumoniae*. Samples from 2014 and 2015 were collected and isolated according to standard procedures recommended by the WHO [18]. Swabs, the total bacterial lawn of the primary gentamicin blood plate, and pneumococcal isolates were frozen at −80 °C in 1 ml of STGG medium.

2.4. DNA isolation

STGG tubes containing the nasopharyngeal swab were thawed on ice and vortexed. 50 μl aliquots were plated onto tryptic soy blood agar plates supplemented with gentamicin using a spreader and incubated overnight at 37 °C in 5% CO_2. 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).

2.5. 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 $\geqslant$70%; co-dominant if >30%, and <70% and minor if $\leqslant$30%.

2.6. Statistical analysis

Statistical significance was assessed using Fisher's exact test. 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].

3. Results

3.1. 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 chil-

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