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Predominance of nontypeable *Haemophilus influenzae* in children with otitis media following introduction of a 3 + 0 pneumococcal conjugate vaccine schedule

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ABSTRACT

In Australia the 7-valent pneumococcal conjugate vaccine (PCV7) is administered at 2, 4 and 6 months of age, with no booster dose. Information on bacterial carriage and the aetiology of recurrent acute otitis media (rAOM) after introduction of PCV7 using the 3 + 0 schedule is required to evaluate the potential impact of second generation pneumococcal vaccines. We found that 2–4 years after introduction of PCV7 in the National Immunisation Program, nontypeable *Haemophilus influenzae* (NTHi) was the predominant pathogen isolated from the nasopharynx and middle ear of children with a history of rAOM. Compared with healthy controls ($n = 81$), NTHi and *Streptococcus pneumoniae* carriage rates were significantly higher in children with a history of rAOM ($n = 186$) (19% vs. 56% $p < 0.0001$ and 26% vs. 41%, $p = 0.02$, respectively). Carriage of PCV7 pneumococcal serotypes was rare, whereas PCV7-related and non-PCV7 serotypes were isolated of 38% of cases and 24% of controls. Serotype 19A was the most common serotype isolated from the nasopharynx and middle ear and accounted for 36% (14/39) of total pneumococcal isolates with reduced susceptibility to cotrimoxazole. Of the 119 children carrying NTHi, 17% of isolates were β-lactamase positive.

The scarcity of PCV7 serotypes in children with and without a history of rAOM indicates that the 3 + 0 PCV7 schedule is preventing carriage and rAOM from PCV7 serotypes. Introduction of new vaccines in Australia with increased pneumococcal serotype and pathogen coverage, including 19A and NTHi, should decrease the circulation of antibiotic-resistant bacteria and reduce the burden of rAOM.

1. Introduction

The global burden of recurrent acute otitis media (rAOM) on the population, health care systems and the economy remains significant, highlighting the need for effective preventative measures [1]. *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) together account for approximately 80% of cases of AOM, with *Moraxella catarrhalis* isolated in 3–20% of cases [2,3]. Colonisation of the nasopharynx with these otopathogens is directly related to the development of rAOM, with colonisation and first AOM episode at a young age being risk-factors for developing rAOM [4,5]. Despite high detection rates of these pathogens in the nasopharynx by bacteriological culture, middle ear effusions (MEE) are often culture-negative due to recent antibiotic treatment and bacterial survival in biofilms [6,7]. Polymerase chain reaction (PCR) provides a sensitive method for detection of non-cultureable bacteria in MEE [8,9].

Introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) at the beginning of this century significantly reduced invasive pneumococcal disease. Trials with this vaccine showed that the efficacy against PCV7-serotype OM was 57%. However, replacement with non-vaccine pneumococcal serotypes and other otopathogens

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such as NTHi has resulted in a limited reduction in overall AOM incidence of 6–9% [10,11]. PCV7 was introduced into the Australian National Immunisation Program for infants at 2, 4 and 6 months of age with no booster dose in 2005 as well as a catch-up program for all children up to 2 years of age at that time. It is important to investigate the bacterial aetiology of rAOM in PCV7-vaccinated children and whether the Australian 3 + 0 vaccine schedule, which is different from the booster schedules used in the UK (2 + 1) or the US (3 + 1), prevents nasopharyngeal carriage and OM from PCV7 serotypes.

Two pneumococcal conjugate vaccines with additional pneumococcal serotypes have recently been developed. PCV7 has been expanded to PCV13 by including 6 additional pneumococcal serotypes (1, 3, 5, 6A, 7F and 19A) [12]. This vaccine was shown to induce comparable immune responses to PCV7 and immunisation with PCV13 resulted in functional anti-polysaccharide antibodies against all 13 serotypes [13]. Pneumococcal nontypeable *H. influenzae* protein D conjugate vaccine (PHiD-CV) is a 10-valent PCV that in addition to the serotypes in PCV7 includes serotypes 1, 5 and 7F with the majority of the polysaccharides conjugated to NTHi Protein D [14]. A clinical trial of an 11-valent predecessor of PHiD-CV demonstrated protection against otitis media caused by *S. pneumoniae* and NTHi, leading to a reduction in NTHi carriage of 39% and NTHi AOM of 35% [15,16].

To estimate the impact of these or other new vaccines on nasopharyngeal carriage and AOM, especially in high-risk individuals, national data are important to ensure that the best vaccination strategy is chosen to give optimal serotype and pathogen coverage that is specific to a geographical area. Therefore, the aim of this study was to investigate nasopharyngeal carriage rates of common otopathogens (*S. pneumoniae*, NTHi, *M. catarrhalis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and β-haemolytic streptococci) in children below 36 months of age and vaccinated with PCV7 at 2, 4 and 6 months, either with a history of rAOM requiring ventilation tube insertion or healthy children with no history of rAOM. MEE specimens from children with rAOM were cultured for the presence of the above otopathogens and PCR detection of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* was conducted to investigate the bacterial aetiology of rAOM in Australia.

2. Patients and methods

2.1. Recruitment of the study cohort

Between November 2007 and May 2009 children below 36 months of age were recruited to this study to investigate the microbiology, immunology and genetics of rAOM (the GROMIT Study). Of these children 98% had received three doses of PCV7 according to the Australian National Immunisation Program at 2, 4 and 6 months of age. Cases were defined as children with a history of at least 3 episodes of AOM requiring insertion of ventilation tubes. Children without rAOM undergoing general surgery (predominantly orthopaedics, strabismus, circumcision, cryptorchidism, hypospadias repair) were recruited as controls. Children with diagnosed immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities and chromosomal or genetic syndromes were excluded. Data on ear disease, vaccination status, antibiotic treatment and host- and environmental risk factors were collected by parental questionnaire and from medical records. The study was approved by the Ethics Committee of Princess Margaret Hospital for Children, Perth, Western Australia and by ethics committees and the institutional boards of hospitals in Perth where recruitment took place. Informed consent was obtained from parents of participating children before recruitment.

2.2. Nasopharyngeal swab collection and processing

Nasopharyngeal swabs were collected while the child was under general anaesthetic for the insertion of ventilation tubes (cases) or minor surgical procedures (controls). A small number of nasopharyngeal swabs (n = 10) from healthy controls were collected by a nurse during visits of healthy control children to the study centre's clinic. A sterile flexible cotton-wool tip swab (Copan, Brescia, Italy) was inserted trans-nasally, reaching the nasopharyngeal space. Swabs were immediately stored in sterile Skim-Milk–Tryptone–Glucose–Glycerol-Broth (STGGB; 3% tryptone soya broth [Sigma–Aldrich, Castle-Hill, Australia], 0.5% glucose, 2% skimmed milk powder and 10% glycerol in 100 mL distilled water), placed on ice and transported to the laboratory within 4 h. Samples were vortexed vigorously for 1 min and stored at −80 °C until analyses. A subset of samples was cultured immediately and after storage at −80 °C, showing no reduction in bacterial viability with storage.

2.3. Middle ear effusion (MEE) collection and processing

MEE samples were collected from anaesthetised children undergoing ventilation tube insertion for rAOM. An anterior-inferior myringotomy incision was made utilising the operating microscope and the MEE sample was collected with a sterile Leukotrap® [Pall corporation, New York, USA] connected to the surgical suction system. The tubing system was rinsed with approximately 1 mL of sterile saline to recover all of the MEE present. The MEE was immediately placed on ice and transported to the laboratory within 4 h where 200 μL of the collected sample was transferred into 1 mL sterile STGGB, vortexed vigorously for 1 min and stored at −80 °C until analyses.

2.4. Bacteriological culture and typing

All nasopharyngeal swabs and MEE specimens were examined for the presence of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *P. aeruginosa*, *S. aureus* and β-haemolytic streptococci using conventional culture methods as previously described [17]. In brief, 10 μL of the nasopharyngeal samples and 100 μL of each MEE sample were streaked onto horse blood agar, chocolate agar (containing 300 mg/L bacitracin, 5 mg/L vancomycin and 0.96 mg/L clindamycin), and colistin nalidixic acid blood agar plates [Oxoid, Australia]. The agar plates were incubated at 37 °C in a humid atmosphere containing 5% CO_2 and inspected at 24 h and 48 h for signs of bacterial growth. Four colonies of presumptive *S. pneumoniae*, two colonies each of NTHi and *M. catarrhalis*, and single colonies of *P. aeruginosa*, *S. aureus* and β-haemolytic streptococci were selected for sub-culture when present. When possible, morphologically distinct colonies of the same species were selected to increase the potential for detection of multiple subtypes. All sub-cultured *S. pneumoniae* isolates were serotyped at the Queensland Pneumococcal Reference Laboratory using type-specific capsular antiserum (Statens Serum Institute, Copenhagen, Denmark). All mucoid *H. influenzae* isolates ($n = 4$) and a 10% sample of non-mucoid isolates were tested with Phadebact *H.influenzae* type b reagent (Bactus AB, Huddinge, Sweden). All presumptive NTHi isolates were further tested by 16S rDNA colony PCR to distinguish true NTHi from non-haemolytic *Haemophilus haemolyticus* [18]. Primers and PCR cycle conditions are given in Table 1. Only true NTHi carriage rates are reported.

2.5. Antibiotic susceptibility testing

S. pneumoniae isolates were tested for susceptibility to oxacillin, chloramphenicol, cotrimoxazole, tetracycline, erythromycin and