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ORIGINAL ARTICLE

What is behind the ear drum? The microbiology of otitis media and the nasopharyngeal flora in children in the era of pneumococcal vaccination

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Aim: This study aims to describe the microbiology of middle ear fluid (MEF) in a cohort of children vaccinated with *Streptococcus pneumoniae* conjugate vaccine (PCV7) having ventilation tube insertion. Nasopharyngeal (NP) carriage of otopathogens in these children is compared with children without history of otitis media.

Methods: Between May and November 2011, MEF and NP samples from 325 children aged <3 years were collected in three major centres in New Zealand at the time of ventilation tube insertion. An age-matched non-otitis-prone comparison group of 137 children had NP samples taken. A questionnaire was completed by both groups.

Results: Immunisation coverage with at least one dose of PCV7 was 97%. *Haemophilus influenzae* was cultured in 19.4% of MEF and was polymerase chain reaction (PCR) positive in 43.4%. *S. pneumoniae* and *Moraxella catarrhalis* were cultured in <10% of MEF samples but were PCR positive for 23.1% and 38.7%, respectively. *H. influenzae* was the most common organism isolated from NP samples (60%) in the grommet group, while *M. catarrhalis* (56%) was the most common in the non-otitis prone group. *S. pneumoniae* was more commonly found in the nasopharynx of children with ear disease (41% vs. 29%). 19F was the most prominent *S. pneumoniae* serotype in NP samples of both groups, but no serotype dominated in MEF. Ninety-five per cent of *H. influenzae* isolates were confirmed to be non-typeable *H. influenzae*.

Conclusion: In this cohort of children with established ear disease requiring surgical intervention, non-typeable *H. influenzae* is the dominant pathogen in both the nasopharynx and MEF.

Key words: *Haemophilus influenzae*; middle ear; otitis media; pneumococcal vaccination; *Streptococcus pneumoniae*.

What is already known on this topic

1 Otitis media, both acute and chronic, is a large burden of disease for young children.
2 Conjugate pneumococcal vaccines impact on invasive pneumococcal disease and pneumonia; however, ear disease impacts are less clear.
3 The nasopharyngeal and middle ear bacterial pathogens are closely linked in children.

What this paper adds

1 Non-typeable *Haemophilus influenzae* is the most common bacterial cause of recurrent acute otitis media and otitis media with effusion in New Zealand children.
2 Nasopharyngeal carriage of otopathogens is more common in children with established ear disease in our population.
3 Despite conjugate vaccination, vaccine-associated pneumococcal serotype 19F remains a common pneumococcal serotype in the nasopharynx of young children and also most likely to carry resistance to antibiotics.

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Otitis media (OM) is one of the most common disorders for which medical care is sought for children. This includes both acute OM (AOM) and OM with effusion (OME). In line with other countries, AOM and OME are a significant burden on the New Zealand (NZ) health-care system and are a common reason for antibiotic prescriptions for young children.[1] Maori and Pacific children in NZ are disproportionately affected with OM, with medical admissions rates for OM-related conditions being twice those of European or other ethnic groups.[1,2] Recurrent AOM (rAOM) and persistent OME are the most common indications for inserting ventilation tubes or 'grommets'.[3]

Streptococcus pneumoniae, non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* are regarded as the main pathogens responsible for middle ear disease.[4–9] The pathogenesis of OM is intricately related to the presence of bacteria colonising the nasopharynx, which provides the reservoir for respiratory pathogens. There is a relationship between nasopharyngeal (NP)-colonising organisms and rAOM.[10–13] The co-colonisation of certain bacteria may also be associated with a higher risk of rAOM.[14] However, the relationship of bacteria in the pathogenesis of OME is not so well established.[12]

Pneumococcal conjugate vaccines (PCVs) significantly decrease the burden of invasive pneumococcal disease such as meningitis but also impact on mucosal disease such as pneumonia and AOM caused by vaccine serotypes.[15] While the efficacy of PCV7 against AOM in early clinical trials was reportedly less than 10%,[16] the efficacy and effectiveness against rAOM and surgical interventions such as ventilation tubes have subsequently been shown to be much greater.[17–19] The impacts of PCV are likely through reduction of NP colonisation by vaccine serotypes which may subsequently reduce OM due to these same vaccine serotypes.[20]

PCV7 was introduced into the NZ national immunisation schedule in September 2008 with catch-up vaccination offered to all infants born after January 2008. In late 2011 (after the completion of this study), the immunisation schedule was revised with replacement of PCV7 with the new 10 valent PCV (PHiD-CV). This change provided a window of opportunity to assess the infectious aetiology of OM following implementation of PCV7 and prior to the change in pneumococcal vaccination to PHiD-CV.

Our study was known at the "OMIVI" (Otitis Media Infectious aetiology & Vaccination Impact) Study. Our aims were to determine the bacteriological causes of rAOM and OME in NZ children. In addition, we wanted to document the NP carriage of organisms known to cause AOM in children with and without a history of rAOM or OME in the context of our changing pneumococcal vaccine schedule.

Methods

Children less than 36 months of age undergoing ventilation tube (grommet) surgery were recruited from three major centres: Starship Children's Hospital (Auckland District Health Board), KidzFirst (Counties Manukau District Health Board) and Christchurch Hospital (Canterbury District Health Board) between May and November 2011 (Fig. 1). These sites represent diverse ethnicities and are the three major referral centres for children requiring surgical intervention for rAOM or OME in NZ. In these centres, the criteria for grommet insertion is >6 episodes of AOM in 12 months or persistent bilateral middle ear effusions for >3 months. Diagnosis at referral was made by trained practitioners, together with micro-otoscopy and tympanometry prior to booking for surgery.

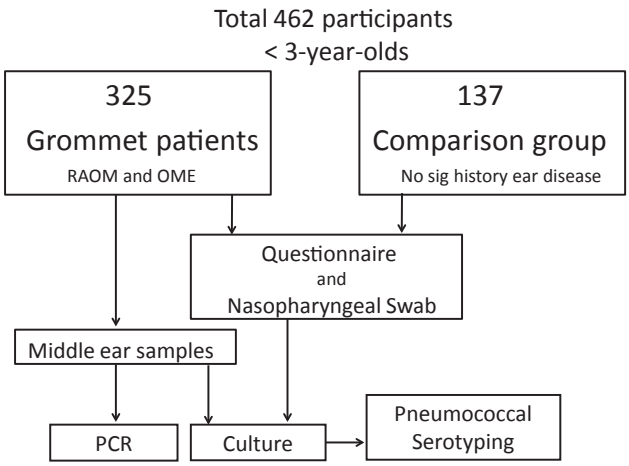

Fig. 1 Otitis Media Infectious aetiology & Vaccination Impact study design.

In each study centre, NP carriage data were also collected on a non-otitis-prone group of children of same age, ethnicity and vaccination status to form our comparison group. On parental history, they had no significant previous ear disease (less than three episodes of AOM in 12 months, no history of OME). These children were having a general anaesthetic for non-ear-related procedures (such as CT/MRI scans, general or non-ear-related surgery). Children with known immune deficiency, cystic fibrosis or craniofacial malformation were excluded from both groups.

Informed consent was obtained by a member of our research team prior to their procedure. Both groups had risk factors for ear disease and epidemiological data collected via parental/carer questionnaire.

All children enrolled were eligible for PCV7 and Hib vaccinations as part of the NZ immunisation schedule. Receipt of vaccinations was confirmed for each child using the National Immunisation Register.

Laboratory method for NP and middle ear samples

NP swabs from both groups were collected as per previously published protocols.[21] In the grommet group, middle ear fluid (MEF) was collected by sterile suction through the myringotomy prior to grommet placement.

Samples were transferred to laboratory within 4 h. MEF were vortexed and 1 mL aliquots stored at −70°C with remaining MEF immediately cultured for bacterial pathogens by standard methods including extended 5-day culture on blood agar to detect *Alliococcus otiditis*.[22]

S. pneumoniae, H. influenzae and *M. catarrhalis* isolates were tested for susceptibility to standard antimicrobials by agar disc

diffusion.[22] Conservative breakpoints for *S. pneumoniae* penicillin susceptibility were used (≤0.06 μg/mL susceptible; 0.12–1 μg/mL intermediate; ≥2 μg/mL resistant). All *S. pneumoniae* had capsular serotyping performed via the Quellung reaction at the National reference laboratory.[23]

For nucleic acid extraction and polymerase chain reactions (PCRs), 200 μL of sample in broth and 5.0 μL of internal control DNA were extracted with EasyMag (BioMerieux, Auckland, New Zealand) generic 2.0.1 protocol. Nucleic acid was recovered in 60 μL of elution buffer. PCRs were based on the Real-time TaqMan (Applied Biosystems Inc., Foster City, CA, USA) PCR format with *S. pneumoniae*[24] performed as a duplex with an internal control (unpublished assay), while *H. influenzae*[25] and *M. catarrhalis*[26] were detected using singleplex PCR assay. Primer and probe sequences are available on request.

Differentiation between *H. influenzae* and *H. haemolyticus* was performed using absence or presence of hpd#3 PCR.[27] Samples identified as *H. influenzae* by culture or PCR were also tested for presence of capsule using bexB PCR.[28]

Ethical approval was obtained from the New Zealand Northern Regional Ethics Committee (NTX/11/04/029).

Statistical analyses

Demographic and response data were compared across centres and study cohorts using odds ratios and *t*-tests. Detection of MEF organisms was analysed with respect to NP organisms using McNemar change test with continuity correction (to allow for matched nature of data). Confidence intervals generated were at 95% level, with *P* value of 0.05 as cut-off. Analysis used R software package version 2.15.2.[29]

Results

Study population

Four hundred and sixty-two children were recruited with 325 in the grommet group and 137 in non-otitis prone group. Our grommet cohort captured 78% of all grommets performed in children in this age group in the three centres over the study period; non-participation was due to missed recruitment and 5% refusing participation. Of the 325 children having grommets, 29% had rAOM, 32% had OME and 37% had rAOM with persistent MEF between acute episodes as indication for surgery. One hundred and thirty-seven children (42%) were estimated by parental history to have had 5–10 episodes of AOM in the last 12 months, with 41 children (13%) estimated to have had >10 episodes. Less than 2% had tympanic membrane retraction as main indication for surgery.

Baseline demographic and clinical data are shown in Table 1. There were no significant differences in age, ethnicity, birthweight, history of breastfeeding, reflux symptoms or

Table 1 Questionnaire data: demographics and otitis media risk factors

	Grommet	Non-otitis prone	*P* values
Total number	325	137	–
Mean age in months (range)	22 (6–36)	21 (3–25)	NS
Male (%)	203 (62%)	98 (71.5%)	NS
Birthweight average: kg (range)	3.39 (0.7–5.2 kg)	3.36 (1.4–4.89)	NS
Gestational age <38 weeks	52/325 (16%)	32/137 (23%)	NS
Child's history of atopy	61/323 (19%)	19/137 (14%)	NS
Family's history of ear disease	155 (48%)	29 (12%)	$P < 0.01$
Ethnicity			
European	58%	45%	$P < 0.05$
Maori	22%	18%	NS
Pacific Island	12%	15%	NS
Other	8%	23%	$P < 0.01$
Vaccination status (%)			
One or more of age-appropriate vaccinations	315 (97%)	134 (97%)	NS
Fully vaccinated	247 (76%)	87 (64%)	$P < 0.01$
Full and 'on time' vaccination†	140 (43%)	41 (30%)	$P < 0.01$
Environmental factors			
Breastfed	281/324 (87%)	116/137 (85%)	NS
Exposure to cigarette smoke	101/324 (31%)	43/136 (31%)	NS
Day care attendance (>4 h/week)	205/322 (64%)	54/134 (40%)	$P < 0.01$
Having any siblings	226/324 (70%)	88/136 (65%)	NS
More than five people in home	59/325 (18%)	23/137 (17%)	NS
Antibiotic use			
Antibiotic use in last month	198/321 (62%)	51/133 (38%)	$P < 0.01$

†'On time' defined as all vaccinations being given within 4 weeks of intended age: 6 weeks, 3 months, 5 months and 15 months. NS, not statistically significant.

number of household occupants between the two groups. Overall, 97% of all participants had received ≥1 dose of PCV7.

Microbiology: MEF

Of the 325 grommet children, 70 children had no MEF bilaterally (21%), 71 had unilateral effusions (22%) and 184 had bilateral effusions (57%). Thus, 255 children had fluid in one or both ears. Of the 441 MEF samples collected, 13 were lost or unable to be processed, giving results from a total of 428 middle ear samples.

One hundred and one children (31%) had a positive culture in one or both ears for one or more of the three main otopathogens. Sixty-three children (19%) had MEF culture positive for *H. influenzae*, 26 (8%) were positive for *S. pneumoniae* and 26 (8%) for *M. catarrhalis*. The majority was culture positive for a single organism (53 grew *H. influenzae* alone, 20 *M. catarrhalis*, 18 *S. pneumoniae*). Ten children (3%) had MEF positive for ≥2 organisms, all having *H. influenzae* as one of the organisms.

The MEF of 66 children (19%) had a positive culture for another organism, of which 23 (7%) were also positive for one of the three main pathogens. Thirty-three children (10%) grew *Alloiococcus otitidis* (11 bilaterally), and five children had *Pseudomonas aeruginosa* cultured from their MEF. Twenty-two children grew presumed skin flora (7%), and six children grew *Staphylococcus aureus* (one of which was methicillin resistant *S. aureus*).

PCR increased identification of presence for the three main otopathogens from 19% (63/325 culture positive of one or more of the three main otopathogens in one or both ears) to 65% (210/325 PCR positive) (Fig. 2). Detection of *H. influenzae* increased from 19% (63/325 children) by culture to 43% (141/324) by PCR. *M. catarrhalis* detection increased from 8% (26/325) to 39% (182/441) and *S. pneumoniae* from 8% to 23%. Two MEF samples had positive culture for *H. influenzae* without concordant positive PCR, and similarly, one sample for *M. catarrhalis* and two samples for *S. pneumoniae*. Sixty-eight children (21%) had two otopathogens detected in their MEF by PCR, and 32 children (10%) had all three otopathogens present. Of the 100 children with more than two organisms detected by PCR present in MEF, 82 had *H. influenzae* present.

Microbiology: NP carriage

There were 137 NP specimens collected from 137 children in the non-otitis prone group and 316 NP specimens collected from the 325 grommet participants. The rates of carriage of otopathogens

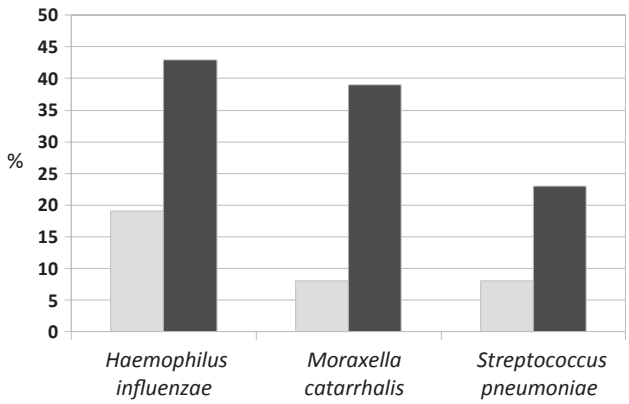

Fig. 2 Culture versus polymerase chain reaction detection of pathogens in Grommet group (*n* = 325): middle ear effusions. □, culture; ■, PCR.

Table 2 Bacterial nasopharyngeal carriage in grommet versus comparison groups

Bacteria	Grommet group *n* = 316 (%)	Comparison group *n* = 137 (%)	*P* value
NP specimen culture positive for one or more pathogens	275 (87)	105 (77)	$P < 0.01$
Haemophilus influenzae: total (%)	195 (62)	58 (42)	$P < 0.05$
Streptococcus pneumoniae: total (%)	135 (43)	40 (29)	$P < 0.05$
VT (PCV7)	36 (11)	11 (8)	NS
NVT	98 (31)	29 (21)	$P < 0.05$
Moraxella catarrhalis: total (%)	181 (57)	68 (50)	NS
NP specimen culture positive for Single pathogen only	102 (32)	58 (42)	$P < 0.05$
NTHi	57 (18)	21 (15)	NS
S. pneumoniae	13 (4)	5 (4)	NS
M. catarrhalis	32 (10)	32 (23)	$P < 0.01$
NP specimen culture positive for Two pathogens	128 (41)	33 (24)	$P < 0.01$
S. pneumoniae + NTHi	30 (9)	11 (8)	NS
S. pneumoniae + *M. catarrhalis*	41 (13)	10 (7)	NS
M. catarrhalis + NTHi	57 (18)	12 (9)	$P < 0.05$
NP specimen culture positive for all Three pathogens: NTHi + *S. pneumoniae* + *M. catarrhalis*	51 (16)	14 (10)	NS

NP, nasopharyngeal; NS, not susceptible; NTHi, non-typeable *H. influenzae*; NVT, *S. pneumoniae* serotypes not included in PCV7; VT, *S. pneumoniae* serotypes included in PCV7.

in the two groups are summarised in Table 2. Culture rates were not statistically different for the three major organisms when compared between ethnic groups.

Correlating NP culture and MEF culture or PCR results

The likelihood that a cultured otopathogen from NP was indicative of the same pathogen in the MEF by culture or by PCR was highly correlated for all three organisms. If culture positive for *H. influenzae* in the NP, a child was >10 times more likely to be culture positive for the same organism in MEF than a child who was culture negative. Similarly, if culture positive for *S. pneumoniae* or *M. catarrhalis* in NP, the child was 15 times more likely to be culture positive for those respective organisms in MEF. The negative predictive value of NP culture for culture negativity of MEF was 96%, 97% and 99%, respectively, for *H. influenzae*, *M. catarrhalis* and *S. pneumoniae*, while the positive predictive values (PPV) were poor (30%, 12% and 18%, respectively). If these otopathogens were cultured from the nasopharynx, the likelihood of the same organism being detected by PCR in MEF had an improved but still poor PPV (61%, 50% and 40%, respectively).

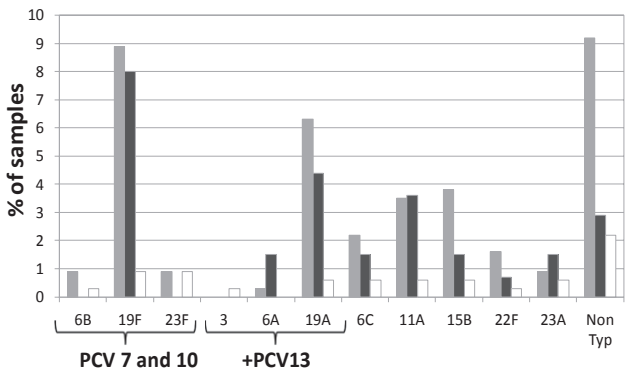

Fig. 3 *Streptococcus pneumoniae* serotypes: culture of nasopharynx and middle ear in grommet group and nasopharynx of comparison group. Only one incongruous result: Ear 23B Nasopharynx 35NT. ▒, grommet nasopharynx (*n* = 134); ■, comparison nasopharynx (*n* = 40); □, grommet middle ear (*n* = 32).

S. pneumoniae serotyping

Of *S. pneumoniae* cultured from NP (*n* = 174), 19F was the most common serotype followed by non-typeable and 19A. Serotyping of the *S. pneumoniae* from MEF (*n* = 32) showed a wide spread of serotypes (Fig. 3). Only one patient had an incongruous serotype in MEF and NP (serotype 35 in NP and 23B in MEF).

Antibiotic susceptibilities

H. influenzae isolates from both MEF and NP were amoxycillin resistant in 20% (16/57 NP comparison group, 31/194 NP grommet group and 20/94 MEF) predominantly due to β-lactamase presence (60/67). Cotrimoxazole resistance was found in 30%, and other antibiotic resistance was uncommon.

Of *M. catarrhalis* isolates, 87% had detectable β-lactamase present, all were sensitive to co-amoxycillin clavulanate, and other resistance was uncommon.

Antibiotic susceptibility data were available in 206 *S. pneumoniae* isolates (Table 3). Reduced susceptibility to penicillin and resistance to cotrimoxazole were most commonly found. Among those with penicillin resistance (minimum inhibitory concentration ≥ 2 μg/mL), 19/21 (90%) were multi-resistant (resistant to ≥3 additional antibiotic classes).

The vast majority of penicillin resistant and multi-resistant isolates were serotype 19F (18/19 isolates) with the remaining one being serotype 19A. Of those with intermediate penicillin susceptibility and multi-resistance, 12/19 were serotype 19F.

Molecular differentiation of *Haemophilus* species

Among the archived *H. influenzae* isolates from NP and MEF, 281 isolates (82% of all *H. influenzae* detected) had extractable nucleic acid available for species differentiation.

Twelve isolates (4%) were identified as *Haemophilus haemolyticus* with negative hpd#3 PCR. One of 12 was from MEF, and the rest was from NP. Of the remaining confirmed *H. influenzae* (269), two were positive for bexB PCR; therefore, likely capsulated Hi and the remaining 99.3% were non-typeable *H. influenzae* (NTHi) (capsule absent). Therefore, of all suspected *H. influenzae*, 95% were NTHi, 4% were *H. haemolyticus* and <1% were capsulated.

Table 3 Antimicrobial resistance and non-susceptibility among *Streptococcus pneumoniae* isolates from grommet and comparison children aged <3 years

	Penicillin*		Cotrimoxazole	Clindamycin	Erythromycin	Tetracycline
	R (%)	NS (%)	R (%)	R (%)	R (%)	R (%)
MEF *n* = 31	2 (6)	7 (23)	6 (19)	6 (19)	5 (16)	4 (13)
NP *n* = 175	19 (11)	57 (33)	56 (32)	32 (18)	41 (23)	40 (23)
Total *n* = 206	21 (10)	64 (31)	62 (30)	38 (19)	46 (22)	44 (21)

*For penicillin: Resistance represents: Minimum Inhibitory Concentration (MIC) > 2. NS represents intermediate plus resistant isolates: MIC > 0.06. R, resistant; NS, not susceptible; MEF, middle ear fluid; NP, nasopharyngeal.

Discussion

Recurrent AOM and OME have a significant disease burden in children younger than 3 years of age in NZ. We describe for the first time microbiology of rAOM and OME in a cohort of NZ children who have received pneumococcal and Hib vaccination. The major strength of this study is that almost 80% of children referred for surgical intervention for OM were captured from the two major NZ cities.

NTHi was the organism most commonly identified from both the NP and MEF in our cohort of children with rAOM or OME. Our findings are consistent by both culture and PCR detection methods and are also in keeping with studies from several other countries where *H. influenzae* has been found to be the most common pathogen isolated in the context of widespread conjugate pneumococcal vaccination.[21,30]

Haemophilus haemolyticus, a non-pathogenic commensal organism, is indistinguishable from *H. influenzae* by conventional microbiology.[27] A further strength in our study was the accurate molecular characterisation[27] to demonstrate over 95% of *H. influenzae* isolates from both NP and MEF samples were truly NTHi. This established an important baseline for monitoring changes rates of *H. influenzae* carriage which could otherwise be masked by *H. haemolyticus* replacing *H. influenzae* in the nasopharynx.

S. pneumoniae was the least commonly detected otopathogen by culture or PCR in MEF or by culture of NP. A wide range of *S. pneumoniae* serotypes were demonstrated in MEF samples with no single serotype that dominated.

In all but one patient, there was a concordance of the *S. pneumoniae* serotype identified in MEF and NP samples, supporting the theory that invasion of the MEF is preceded by colonisation of the NP. Similarly, NP culture positivity for other otopathogens (*H. influenzae* or *M. catarrhalis*) showed a child to be much more likely to be culture positive in MEF than a child not carrying those organisms. The high negative predictive values for all three organisms may enable a negative NP culture in OME or rAOM cases to help rule out the likelihood of organisms in the MEF.[31]

The pneumococcal serotypes most frequently found on NP samples in both the grommet and comparison groups were 19F (PCV7 serotype) and 19A (non-PCV7 serotype). Persisting carriage of vaccine 19F serotype in our vaccinated population (>70% fully vaccinated) may be due to several factors. Although PCV7 was introduced in NZ 3 years prior to our study, improved immunisation rates and timely (age-appropriate) vaccine administration have been more gradually achieved as a priority national health target; this may have slowed the herd immunity impact of PCV7.[32]

Serotype 19F was most associated with multi-resistance and penicillin resistance. Reduced penicillin susceptibility was seen to a much lesser extent with serotype 19A. This is in contrast to other countries where 19A is commonly multi-drug resistant in PCV7-vaccinated and unvaccinated populations.[33,34] Both the grommet and non-otitis prone groups had high rates of antibiotic pre-treatment (50% received antibiotics in last month). This high antibiotic exposure in our cohort may have selected for persistence of NP carriage of drug-resistant 19F serotype despite vaccination.

Despite relatively high NP carriage of 19F and 19A serotypes, these serotypes were infrequently isolated from MEF. A global review of studies following introduction of PCV7 has suggested that serotypes 3, 6 and 19A may be increasingly important in AOM.[35] In our study, high prevalence of 19F and 19A in the NP was not reflected by higher rates of these serotypes in the MEF, suggesting these serotypes showed NP colonisation with a low rate of invasion into the middle ear cleft in child with established ear disease. However, small numbers of culture-positive *S. pneumoniae* from MEF limit conclusions around dominant serotypes in our cohort.

Evaluation of otopathogens and their antimicrobial patterns may have implications for empiric treatment of recurrent AOM. In our study, a significant proportion of *M. catarrhalis*, and to a lesser extent *H. Influenzae*, produced beta-lactamase and was resistant to amoxycillin. However, both comparison and cases were likely to have received antibiotics in the prior month (40% and 60%, respectively) which along with vaccination status will impact upon NP organisms and resistance profiles.

In our cohort, NP carriage of one or more otopathogens was more likely in the grommet children. Studies have described the carriage of multiple otopathogens in the NP to be significantly associated with presence of bacteria in MEF.[36] We have shown when *H. influenzae* and *S. pneumoniae* co-colonise the NP, *H. influenzae* is more likely to be identified in MEF.[14]

Conclusion

This study represents the most comprehensive national data collection on the microbiology of middle ear disease in NZ children and demonstrates that NTHi is the most commonly isolated organism in established ear disease.

The 10 valent PCV (PHiD-CV) was included in the NZ national immunisation schedule in 2011[37,38] and has the potential to reduce the impact of NTHi infections including OM. This is due to the conjugation of pneumococcal polysaccharide antigens to the Protein D component of *H. influenzae*. We aim to repeat data collection in 2014 with a cohort of children who have been vaccinated with PHiD-CV. This will enable us to compare the infectious aetiology of OM and NP carriage of OM pathogens in both the PCV7- and PHiD-CV-vaccinated cohorts.

Acknowledgements

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