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Microbial profiling does not differentiate between childhood recurrent acute otitis media and chronic otitis media with effusion

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ABSTRACT

Objectives: Otitis media (OM) is one of the most frequent diseases of childhood, with a minority of children suffering from recurrent acute otitis media (rAOM) or chronic otitis media with effusion (COME), both of which are associated with significant morbidity. We investigated whether the microbiological profiling could be used to differentiate between these two conditions.
Methods: Children up to five years of age, with rAOM ($n = 45$) or COME ($n = 129$) and scheduled for tympanostomy tube insertion were enrolled in a prospective study between 2008 and 2009. Middle ear fluids ($n = 119$) and nasopharyngeal samples ($n = 173$) were collected during surgery for bacterial culture and PCR analysis to identify *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*, and to detect 15 distinct respiratory viruses.
Results: The occurrence of bacterial and viral pathogens in middle ear fluids did not significantly differ between patients suffering from rAOM and COME. In both patient cohorts, *H. influenzae* and rhinovirus were the predominant pathogens in the middle ear and nasopharynx. Nasopharyngeal carriage with two or three bacterial pathogens was associated with the presence of bacteria in middle ear fluid ($P = 0.04$). The great majority of the bacteria isolated from middle ear fluid were genetically identical to nasopharyngeal isolates from the same patient.
Conclusions: Based on these results, we propose that the common perception that rAOM is associated with recurrent episodes of microbiologically mediated AOM, whereas COME is generally a sterile inflammation, should be reconsidered.

1. Introduction

Otitis media (OM) is one of the most frequent diseases during childhood and the most common reason for young children to visit a physician. In many countries, it is the most common reason for children to receive antibiotics or to undergo surgery [1–3].

OM is a common denominator for a variety of middle ear diseases that can be divided into various categories, including acute otitis media (AOM) and otitis media with effusion (OME) [4]. AOM is defined as the presence of middle ear effusion accompanied by signs of acute inflammation of the middle ear, such as otalgia, otorrhea, fever, and malaise or irritability of the child [3,5]. OME, on the other hand, can either develop as a sequel to AOM, or develop de novo, the primary symptom being hearing loss due to the presence of middle ear fluid in the middle ear cavity (but in the absence of signs of acute inflammation) [6]. Albeit often self-limiting, in 10–20% of the cases OM can result in chronic OME (COME) or recurrent AOM (rAOM) disease [7].

Although there is currently no universal standard for OM management, several options are available to the clinician, namely: watchful waiting, antibiotic treatment, adenoidectomy, ventilation tube insertion, or vaccination. AOM management generally involves adequate analgesics with an optional observation period for 48–72 h [5]. Thereafter, antibiotic treatment or, in the case of recurrent infections, antibiotic prophylaxis or ventilation tube insertion are considered [5,8,9]. In contrast, for OME disease, medical intervention is appropriate only if persistent

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clinical benefits can be achieved in the absence of spontaneous resolution. Therefore, healthy children with OME are observed for at least 3 months before medical intervention is considered, in which case surgical treatment involving the insertion of ventilation tubes or adenoidectomy is feasible [3,10]. Vaccination against OM diseases is currently very limited, being directed against very few bacterial OM pathogens, and only one of the viral OM pathogens, i.e. the influenza vaccine. The heptavalent pneumococcal conjugate vaccine is primarily directed against pneumococcal invasive disease, whereas the introduction of vaccination only reduced the overall OM incidence by 6–9% due to serotype or pathogen replacement [11]. However, herd immunity may induce a further decline in OM incidence, in line with nasopharyngeal colonization studies 3 years after the introduction of the vaccine [12,13].

A key element in the pathogenesis of OM is the nasopharynx, as this niche is the reservoir for bacterial pathogens involved in middle ear infections [4]. Colonization of the nasopharynx with *Streptococcus pneumoniae*, *Haemophilus influenzae* or *Moraxella catarrhalis* (the three most important bacterial pathogens associated with OM disease) at an early stage, has been shown to predispose children for development of rAOM [14]. In addition, OM-prone children have increased carriage rates of these bacterial pathogens compared to healthy controls [15,16]. In AOM, *S. pneumoniae* is the most frequently detected pathogen in middle ear fluid, followed by non-typeable *H. influenzae* and *M. catarrhalis* [17–19]. However, *H. influenzae* tends to predominate in COME, followed in a lesser extent by *S. pneumoniae* and *M. catarrhalis* [20]. In general, bacteria have been found less frequently in the middle ear of children suffering from COME compared to AOM (approximately 30% versus 70%, respectively) [17].

In addition, many studies over the past decades have shown a close association between AOM and respiratory viral infections [21–24]. Viral upper respiratory tract infections (URI) predispose children to AOM, as infection may cause Eustachian tube dysfunction, and facilitate an increase in adherence of bacteria to epithelial cells, resulting in a rise in bacterial colonization of the nasopharynx and a modulation of the host's immune function [25–27]. Nevertheless, studies describing specific associations between respiratory viruses and bacteria in rAOM and COME are scarce.

In this study, we investigated bacterial and viral colonization and infection in the middle ear and nasopharynx of children diagnosed with rAOM or COME. In particular, the contribution of 3 major bacterial pathogens (*S. pneumoniae*, *H. influenzae* and *M. catarrhalis*) in the absence or presence of 15 distinct respiratory viruses was studied in relation to rAOM and COME disease.

2. Materials and methods

2.1. Study design

A cohort of children up to five years of age who suffered from rAOM or COME was examined in this prospective clinical study. The cohort was enrolled at a secondary and a tertiary care hospital in Nijmegen, the Netherlands, from April 1st 2008 to July 1st 2009, and included children suffering from rAOM or COME who underwent surgery for the insertion of ventilation tubes. Recurrent AOM was defined as 3 or more episodes of AOM in the last 6 months or 4 episodes in the last 12 months [28]. The COME patient population consisted of children who had experienced a period of persistent OM with effusion lasting longer than 3 months. RAOM or COME diagnosis was made by an otolaryngologist in routine clinical practice based upon signs, symptoms, otoscopy and audiometry including tympanometry. Patients with a history of malignancy, organ transplantation or immune deficiency were excluded from participation, as well as patients with recent elective ear surgery or systemic infectious diseases. Children with AOM and/or fever ($T \geq 38\ ^\circ C$) at the time of ventilation tube insertion were rescheduled for the procedure. Adenoidectomy performed in the same surgical setting was not considered to be an exclusion criterion. Patient characteristics and risk factors were acquired using a questionnaire, collected at the day of ventilation tube insertion. Ethical permission was obtained from the Committee on Research Involving Human Subjects in January 2008 (CMO 2007/239, international trial register number: NCT00847756).

2.2. Clinical samples

Per child, one middle ear fluid sample and one nasopharyngeal sample was collected. Middle ear fluid was collected during surgery using a middle ear fluid aspiration system (Kuijpers Instruments, Groesbeek, The Netherlands) [29], nasopharyngeal samples, taken through the nose, were obtained using a cotton wool swab (192C, Copan, Brescia, Italy). Middle ear fluid was suspended in 2 ml saline and divided into aliquots for bacterial culture, bacterial PCR, and viral multiplex PCR. The nasopharyngeal samples were used for bacterial culture and thereafter stored at $-80\ ^\circ C$ in 1 ml of 80% glycerol for subsequent viral analysis.

2.3. Microbiology

Middle ear fluid and nasopharyngeal samples were cultured directly after collection according to standard laboratory procedures [14] to determine the presence of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*. Thereafter, bacteria were stored at $-80\ ^\circ C$ in appropriate media containing an additional 15% (*S. pneumoniae*, *H. influenzae*) or 50% glycerol (*M. catarrhalis*). The less frequently described otopathogens *Staphylococcus aureus*, *Streptococcus pyogenes*, *Haemophilus parainfluenzae*, *Pseudomonas aeruginosa* and *Alloicoccus otitidis* were also included in the analysis if detected during bacterial culture.

To further characterize the bacterial isolates, all *S. pneumoniae* and *H. influenzae* isolates were cultured overnight on, respectively, blood agar and chocolate agar plates at 37 °C supplemented with 5% CO_2. Pneumococcal isolates were serotyped using the Quellung reaction (Statens Serum Institute, Copenhagen, Denmark) and multiplex PCR as described previously [30]. *H. influenzae* isolates were serotyped using slide agglutination according to the manufacturer's instructions (Becton–Dickinson, Breda, The Netherlands) [31].

S. pneumoniae, *H. influenzae* or *M. catarrhalis* obtained from middle ear fluid, as well as the equivalent pathogen isolated from the nasopharynx of the same patient, were further analyzed using multilocus sequence typing (MLST). Genomic DNA was isolated using a Qiagen Genomic-tip 20/G Kit (Venlo, The Netherlands) according to the manufacturer's instructions. DNA amplification of the MLST loci and sequencing was performed as described previously [32–35]. Quantitative DNA analysis of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* by real-time PCR was performed as previously described. The respective genes chosen for bacterial quantification were the *S. pneumoniae ply* gene [36], *H. influenzae* 16S rRNA gene [37] and the *M. catarrhalis ompJ* gene [38].

2.4. Virology

Middle ear fluid and nasopharyngeal samples were analyzed by multiplex PCR as previously described [39]. Briefly, upon thawing, nucleic acids were extracted from each sample using the MagNA Pure LC System and the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Applied Science, Almere, The Netherlands) according to manufacturer's instructions. A multiplex real-time PCR assay

targeting 15 different viral pathogens was used. This assay was designed for the detection of the specific viral genomes of influenza virus (IV) type A and B, coronavirus (CoV) 229E and OC43, human bocavirus (hBoV), enterovirus (EV), adenovirus (AdV), parechovirus (PeV), parainfluenza virus (PIV) types 1–4, human metapneumovirus (hMPV), rhinovirus (RV) and respiratory syncytial virus (RSV). An internal control consisting of phocine herpesvirus (PhPV, IC DNA control) and equine arthritis virus (EAV, IC RNA control) was included in the assay. RNA was reverse transcribed to cDNA using the TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Nieuwekerk aan de IJssel, The Netherlands) in a 50 μl reaction mix containing 20 μl of nucleic acid isolate and random hexamers as primers (2.5 μM), according to the manufacturer's instructions. PCRs were performed on the LightCycler 480 instrument using LightCycler 480 Probes Master Mix (Roche Diagnostics). Validated primer/probe-mixes were purchased from Diagenode (Liège, Belgium) and used according to the manufacturer's instructions. Cycling conditions were 95 °C for 5 min, followed by 50 cycles of 95 °C for 15 s and 55 °C for 15 s and 72 °C for 20 s.

2.5. Statistical analysis

Statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 (SPSS Inc., Chicago, IL, USA). The chi-square test or Mann–Whitney *U*-test was used when appropriate to calculate the statistical differences between patient baseline characteristics. The chi-square test was used in the analysis of categorical data obtained from bacterial culture, bacterial PCR and viral PCR. A *P*-value <0.05 was considered statistically significant.

3. Results

3.1. rAOM and COME cannot be differentiated based on bacterial infection

A total of 176 children were enrolled in the study (age range 0.6–5.9 years). Selected demographic and clinical characteristics of the cohort are shown in Table 1. Recurrent AOM was mostly diagnosed at a younger age, while the prevalence of COME increased with age (rAOM 75% during the first year of life vs. 17% at 5 years of age; COME 25% during the first year of life vs. 83% at 5 years of age). A middle ear fluid specimen was collected from 69% of the children ($n = 119$), with a significant difference in the presence of middle ear fluid and OM diagnosis (rAOM $n = 26$ (58%) vs. COME $n = 97$ (75%), $P = 0.02$). Children suffering from rAOM presented with less ENT-related surgery in their medical history when compared to children suffering from COME, but received significantly more antibiotics in the year prior to surgery.

3.2. Microbial profiling does not differentiate between rAOM and COME

The frequency of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in middle ear fluid and the nasopharynx is shown in Table 2. In total, 117 samples were screened for bacterial presence by both, bacterial culture and PCR. Not surprisingly, PCR detected bacterial DNA in a statistically significant percentage of culture-negative middle ear effusions: *S. pneumoniae* in 4.1% (NS), *H. influenzae* in 16.5% ($P < 0.01$) and *M. catarrhalis* in 19% ($P < 0.01$).

Importantly, the presence of each specific pathogen, measured using conventional culture techniques or PCR, was not significantly different between the rAOM and COME cohorts (Table 2).

Table 1
Characteristics of the pediatric study population.

	rAOM $n = 25$ (%)	COME $n = 94$ (%)	*P*-value
Male sex	17/25 (69)	52/94 (55)	0.09
Mean age, years [SD]	3.3 [1.69]	4.02 [1.43]	0.10
<2 years of age	7 (28)	13 (14)	0.09
Presence of middle ear fluid (MEF)[a]	25/25 (100)	94/94 (100)	-
≥4 URTI in the preceding year[b]	8/22 (36)	21/86 (24)	0.26
Recent antibiotic use	**9/25 (36)**	**9/94 (10)**	**<0.01**
History of ENT surgery	8/18 (44)	37/67 (55)	0.42
Asthma/wheezing	2/23 (87)	7/87 (8)	0.92
Allergic rhinitis	2/22 (9)	4/86 (5)	0.42
Eczema	3/23 (13)	11/86 (13)	0.97
Birth weight, mean, (g, [SD])	3291 [624]	3308 [791]	0.82
Breast feeding <3 months	15/23 (65)	51/87 (59)	0.57
PCV7 immunization[c]	**11/25 (44)**	**20/94 (21)**	**0.02**
Tobacco smoke exposure	8/21 (38)	26/84 (31)	0.53
Older siblings	11/18 (61)	47/74 (64)	0.85
Day care attendance	10/22 (46)	40/84 (48)	0.86

Significant differences are marked in **bold**.
[a] The MEF of 4 patients could not be collected for bacterial culture.
[b] Upper respiratory tract infections.
[c] The **PCV7** vaccine was introduced in Dutch National Immunization Program for children born after 01-04-2006.

3.3. The presence of multiple bacterial species of middle ear fluid was sporadic

The presence of multiple bacterial species within middle ear fluids (based on bacterial culture) was sporadic: co-infection by *S. pneumoniae* and *H. influenzae* was observed in a single patient only, as was co-infection by *H. influenzae* and *M. catarrhalis*.

Based on the PCR results, the presence of multiple bacterial pathogens was sporadic and similar in rAOM (10%) and COME (15%). In rAOM *S. pneumoniae* and *M. catarrhalis* were both detetected in 1 child (4%), *H. influenzae* and *M. catarrhalis* in 2 children (8%), and all 3 pathogens in 1 child (4%). In children suffering from COME, *S. pneumoniae* and *M. catarrhalis* were both detected in 3 children (3%), *M. catarrhalis* and *H. influenzae* in 5 children (5%), and *S. pneumoniae* and *H. influenzae* in 2 children (2%).

3.4. Bacterial nasopharyngeal carriage is associated with bacterial presence in the middle ear

H. influenzae was the most frequently detected bacterial pathogen in the nasopharynx and was equally present in both the rAOM and COME cohort (68% versus 70%, respectively, Table 2). Colonization of the nasopharynx by *S. pneumoniae* occurred in 44%

Table 2
Frequency of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in middle ear fluid (MEF) and the nasopharynx of children suffering from rAOM and COME.

	Bacterial culture MEF		Bacterial PCR MEF		Bacterial culture nasopharynx	
	rAOM $n = 25$	COME $n = 94$	rAOM $n = 24$	COME $n = 93$	rAOM $n = 25$[a]	COME $n = 94$[a]
No bacteria, *n* (%)	18 (72)	64 (68)	9 (38)	45 (48)	2 (8)	9 (10)
S. pneumoniae, *n* (%)	0 (0)	5 (5)	3 (13)	6 (7)	11 (44)	57 (61)
H. influenzae, *n* (%)	6 (24)	18 (19)	10 (42)	30 (32)	17 (68)	66 (70)
M. catarrhalis, *n* (%)	1 (4)	9 (10)	7 (29)	22 (24)	12 (48)	40 (43)

[a] MEF positive patients only.

of the rAOM patients, compared to 61% of COME patients, while *M. catarrhalis* was detected in the nasopharynx of 48% of the rAOM and 43% of the COME patients. Eight percent and 10% of the children were culture negative for nasopharyngeal carriage in rAOM and COME disease, respectively (Table 2). Of note, co-colonization of two or more bacterial pathogens in the nasopharynx was significantly associated with the presence of bacteria in middle ear fluid ($P = 0.04$).

3.5. Bacteria in the nasopharynx and middle ear are often genetically identical

The presence of *S. pneumoniae* or *H. influenzae* in middle ear fluid correlated positively with the presence of the same species in the nasopharynx (*S. pneumoniae*, $P = 0.05$; *H. influenzae*, $P = 0.04$). *M. catarrhalis* was found in the nasopharynx of 42% of the children, with simultaneous occurrence of *M. catarrhalis* in the middle ear in 8% of the children; this latter association was not significant.

Serotype analysis of the 5 *S. pneumoniae* isolates cultured from middle ear fluid revealed the following serotypes: 23A ($n = 1$), 23F ($n = 1$), 6B ($n = 1$), 19A ($n = 1$) and 7F ($n = 1$). In all cases, the corresponding serotype was also found in the nasopharynx. No statistically significant differences were observed between the 68 nasopharyngeal *S. pneumoniae* isolates, regarding pneumococcal vaccine types (i.e. those present in heptavalent Pneumococcal Conjugate Vaccine) and non-vaccine types (NVT, rAOM 72%; COME, 69%), nor between typeable and non-typeable (NT) pneumococcal strains (NT: rAOM 9%; COME 2%) when comparing rAOM and COME cohorts.

Twenty-two of the *H. influenzae* isolates cultured from the middle ear were non-typeable (rAOM 83%; COME 100%), compared to 80 non-typeable isolates cultured from the nasopharynx (rAOM 95%; COME 94%). Serotype E was detected in 4% of the middle ear fluid and 4% of the nasopharynx specimens. Serotype B was detected in 1 nasopharyngeal specimen.

MLST analysis revealed that the majority of nasopharyngeal and middle ear fluid isolates obtained from the same child were genetically identical (*S. pneumoniae* and *M. catarrhalis* 100%; *H. influenzae* 80%). Further, no clonal relationships were observed for any of the 3 bacterial pathogens, i.e. there was no evidence to suggest that isolates originated from a single "founder" isolate spreading throughout the community when the respective isolates were compared to their pathogen-specific databases.

3.6. Rhinovirus dominates in both rAOM and COME and contributes to bacterial colonization

Rhinovirus was the most frequently detected virus in middle ear fluids in both patient cohorts, followed by enterovirus, coronavirus and parainfluenza viruses (Table 3). The presence of enterovirus in middle ear fluids was associated with the presence of rhinovirus, as 8 out of the 9 enterovirus-positive middle ears were also positive for rhinovirus ($P = 0.01$). Nevertheless, in the vast majority of the virus-positive samples only a single virus was detected.

Rhinovirus was also the most frequently detected virus in the nasopharynx, followed by enterovirus, parainfluenza viruses 1–4 and coronavirus (Table 3). Nasopharyngeal colonization with enterovirus was significantly more frequent in rAOM patients than in COME patients ($P = 0.04$). In contrast to the results obtained from middle ear fluids, no association between rhinovirus and enterovirus could be found in the nasopharynx. Similar to middle ear fluid, in the majority of the virus-positive nasopharyngeal samples only a single virus was detected (74%).

Interestingly, a significant association was observed for bacterial-viral co-occurrence in the nasopharynx. In more detail, the presence of a virus was accompanied by the presence of a bacterium in 92% of the virus positive nasopharyngeal samples, whereas virus was present in 74% of the bacterial positive nasopharyngeal samples ($P = 0.02$). More specifically, the presence of *S. pneumoniae* and *H. influenzae* was significantly associated with the presence of rhinovirus ($P = 0.05$ and $P < 0.01$, respectively), and the presence of *M. catarrhalis* was significantly associated with the presence of enterovirus ($P = 0.02$). However, no significant difference in bacterial-viral co-colonization of the nasopharynx was detected between children with rAOM or COME.

Table 3
Presence of viruses in middle ear fluid and the nasopharynx of children suffering from rAOM and COME.

	Middle ear fluid		Nasopharynx	
	rAOM $n = 25$ (%)	COME $n = 86$[b] (%)	rAOM[a] $n = 22$[c] (%)	COME[a] $n = 81$[c] (%)
No viruses detected, *n* (%)	10 (40)	40 (47)	5 (23)	23 (28)
Rhinovirus, *n* (%)	11 (44)	38 (44)	11 (50)	43 (53)
Enterovirus, *n* (%)	2 (8)	7 (8)	**5 (23)**	**6 (7)**
Coronavirus, *n* (%)	2 (8)	2 (2)	1 (5)	3 (4)
Parainfluenzavirus, 1–4, *n* (%)	1 (4)	1 (1)	2 (9)	8 (10)
Others, *n* (%)[d]	0 (0)	7 (8)	4 (18)	15 (19)

Significant differences are shown in **bold**. **P = 0.04**.
[a] MEF positive patients.
[b] The MEF of 8 COME patients could not be collected for viral PCR.
[c] The nasopharyngeal swab of respectively 3 rAOM and 13 COME patients could not be used for viral PCR.
[d] Adenovirus, influenzavirus type A and B, hMPV, hPeV, hBV, RSV.

4. Discussion

In this prospective study, we investigated whether the clinical signs and symptoms specific for rAOM and COME could be microbiologically differentiated by the presence of bacteria and viruses in middle ear fluid. The results of our study show that the most predominant bacterial pathogen associated with middle ear fluids obtained from children suffering from rAOM and COME is non-typeable *H. influenzae*, and that the presence of *S. pneumoniae*, *H. influenzae* or *M. catarrhalis* is not significantly different between children with rAOM or COME. With respect to the nasopharynx (and similar to the results obtained using middle ear fluids), the most frequent bacterial pathogen detected within both patient cohorts was non-typeable *H. influenzae*. The predominance of *H. influenzae* in the middle ear and nasopharynx of rAOM and COME patients is similar to other otitis media studies performed elsewhere [40,41].

MLST analysis of the isolates indicated that the pathogens present in the nasopharynx and middle ear were genetically identical within the same child. The *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* genotypes isolated from the total cohort of children comprised a genetically heterogeneous population structure, i.e. the isolates were genetically highly diverse, even though they all originated from a relatively restricted geographical region of The Netherlands. Importantly, the bacterial pathogens that were detected in the middle ear fluids of our cohorts tended to be genetically identical (using MLST) to those isolates cultured from the nasopharynx (80–100%), a finding that correlates with previously published data. Studies investigating the genetic relatedness of bacteria obtained from different locations of the upper respiratory tract, and within a single patient are scarce. Various approaches to determine the genetic relatedness of nasopharyngeal and OM isolates have been described, however MLST was only used once, a technique that is considered to be the gold standard regarding bacterial genotyping [42–44].

Rhinoviruses have been implicated in the pathogenesis of OM [45,46] and showed to be the most frequently found virus in the nasopharynx and middle ear of both COME and rAOM patients. Co-infection of rhinovirus and enterovirus was found in the middle ear, but not in the nasopharynx. Co-occurrence of these viruses has been previously described by Rezes et al., who found rhinovirus in middle ear fluid in 4.8% of the 17 children, enterovirus in 23.8% and both rhino- and enterovirus in 14.3% [46]. Nevertheless, in our study the co-occurrence of multiple viruses in middle ear fluid or in the nasopharynx was rare. In the nasopharynx of our OM patients we found a single virus in 46% and 2 viruses in 9%, in contrast to healthy children as described by Bogaert et al. [47]. They found two or more viruses in the majority of the virus positive nasopharyngeal samples, in 96 children up to two years of age [47]. In our cohort only 15% of the children under the age of 2 had maximal two viruses in the nasopharynx. This finding suggests that in symptomatic children suffering from OM one virus is predominant in the nasopharynx, whereas in healthy children more viruses can be present without clinical significance.

The ability of upper respiratory tract viruses to facilitate secondary bacterial infection in AOM has been described extensively in the literature [27,48,49]. Influenza A and *S. pneumoniae*, RSV and *H. influenzae* are two examples of viral-bacterial interaction investigated in both animal models and human studies [50]. To our knowledge this is the first report describing a highly significant association for the co-occurrence of bacteria and virus in the nasopharynx of rAOM and COME patients. More specifically, nasopharyngeal co-colonization of rhinoviruses with either *S. pneumoniae*, or *H. influenzae*, was statistically significant. Pitkaranta et al. observed a positive correlation between the presence of *S. pneumoniae* or *M. catarrhalis* and rhinovirus in the nasopharynx, but not of *H. influenzae* and rhinovirus [45]. However, the authors focused on nasopharyngeal samples of OM-prone children, and exclusion criteria for their study were either recent or expected ventilation tube insertion and COME diagnosis [45]. In the present study, no association between the co-occurrence of these viruses and bacteria was observed in middle ear fluids for both rAOM and COME cohorts, likely caused by the relatively low number of bacteria detected in middle ear fluid. In addition, since viruses can predispose to bacterial super-infection, it is possible that they are already cleared from the middle ear cavity at the time of surgery.

There are a few limitations of this study. First, the number of children with rAOM enrolled in this study is limited. Second, although we used internationally accepted definitions for rAOM and COME, the timing of clinical sampling in relation to the exact onset of OM is unknown. Finally, while bacteria can colonize and persist in the human respiratory tract for months, infection with respiratory viruses results in acute viral replication, which is cleared within days to weeks. As a consequence, the persistence of bacterial DNA probably exceeds that of viruses.

In conclusion, we performed a prospective cohort study to investigate the frequency and types of bacterial and viral pathogens in the middle ear and nasopharynx of children suffering from rAOM or COME. Our results show that the same pathogenic bacterial species and viruses are implicated in the pathogenesis of both rAOM and COME disease. Further, our findings do not support the general assumption that the microbial profile is pathognomonic for either rAOM or COME, or that COME is merely a consequence of persistent sterile inflammation in the middle ear.

Conflicts of interest

All authors declare there is no conflict of interest.

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