High Detection Rates of Nucleic Acids of a Wide Range of Respiratory Viruses in the Nasopharynx and the Middle Ear of Children With a History of Recurrent Acute Otitis Media

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Both bacteria and viruses play a role in the development of acute otitis media, however, the importance of specific viruses is unclear. In this study molecular methods were used to determine the presence of nucleic acids of human rhinoviruses (HRV; types A, B, and C), respiratory syncytial viruses (RSV; types A and B), bocavirus (HBoV), adenovirus, enterovirus, coronaviruses (229E, HKU1, NL63, and OC43), influenza viruses (types A, B, and C), parainfluenza viruses (types 1, 2, 3, 4A, and 4B), human metapneumovirus, and polyomaviruses (K1 and WU) in the nasopharynx of children between 6 and 36 months of age either with (n = 180) or without (n = 66) a history of recurrent acute otitis media. The co-detection of these viruses with Streptococcus pneumoniae, nontypeable Haemophilus influenzae, and Moraxella catarrhalis was analyzed. HRV (58.3% vs. 42.4%), HBoV (52.2% vs. 19.7%), polyomaviruses (36.1% vs. 15.2%), parainfluenza viruses (29.4% vs. 9.1%), adenovirus (25.0% vs. 6.1%), and RSV (27.8% vs. 9.1%) were detected significantly more often in the nasopharynx of children with a history of recurrent acute otitis media compared to healthy children. HRV was predominant in the middle ear and detected in middle ear effusion of 46% of children. Since respiratory viruses were detected frequently in the nasopharynx of both children with and without a history of recurrent acute otitis media, the etiological role of specific viruses in recurrent acute otitis media remains uncertain, however, anti-viral therapies may be beneficial in future treatment and prevention strategies for acute otitis media.

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INTRODUCTION

Otitis media is one of the most common infectious diseases in childhood and the main reason for physician visits, antibiotic prescriptions and surgery in children, thereby causing a significant burden on the healthcare system and the economy [Rovers et al., 2004]. In acute otitis media, middle ear effusion is present behind the tympanic membrane with signs of middle ear inflammation. The presence of middle ear...
effusion without acute inflammation, either persisting after acute otitis media or occurring de novo, is defined as otitis media with effusion. Although acute otitis media is generally considered to be a bacterial infection, it is acknowledged that respiratory viruses play a role in the development of acute otitis media by increasing the adherence of bacteria to epithelial cells and by virus-induced dysfunction of the Eustachian tube, which interferes with clearance of middle ear effusion. In addition, host-mediated responses to viral infections such as production of cytokines and inflammatory mediators may contribute to the pathology of otitis media [Heikkinen and Chonmaitree, 2003; Nokso-Koivisto et al., 2006]. The persistence of viruses in the middle ear may also lead to relapse of acute otitis media, even if the initial acute otitis media episode was cleared, and may therefore contribute to the long-term presence of middle ear effusion [Rovers et al., 2004].

Using culture methods, viruses have been found in the nasopharynx of 30–50% of children with acute otitis media and in 10–20% of middle ear effusion specimens [Heikkinen and Chonmaitree, 2003]. The use of more sensitive PCR techniques and the ability to detect novel viruses such as human bocavirus (HBoV), polyomaviruses (PyV), human metapneumovirus, and human rhinovirus C (HRV C) described recently has significantly increased these percentages, with one study indicating that a virus was present in the nasopharynx of approximately 90% of children with acute otitis media [Heikkinen and Chonmaitree, 2003]. Studies showing protection against acute otitis media development by immunization with influenza vaccines and the demonstration that experimental viral infection of healthy volunteers resulted in otologic changes, further suggest a role for viruses in acute otitis media pathogenesis [Buchman et al., 2002; Marchisio et al., 2009]. Data is inconsistent regarding the importance of specific viruses in acute otitis media. In studies using viral culture and antigen detection methods, respiratory syncytial virus (RSV) was the most prevalent virus in the nasopharynx and middle ear of children with acute otitis media, whereas a study using PCR assays indicated that human rhinovirus (HRV) was predominant [Pitkaranta et al., 1998; Heikkinen et al., 1999; Yano et al., 2009]. To gain a better understanding of the potential role for specific viruses in otitis media, in 238 middle ear effusion samples collected from 143 children with persistent middle ear effusion due to recurrent acute otitis media. The association of the presence of specific viruses in the nasopharynx and the middle ear of children with a history of recurrent acute otitis media was investigated to determine the capacity of specific viruses to enter and/or persist in the middle ear. In addition, specific associations between viral and bacterial pathogens were investigated. Together this data will further the understanding of the potential role for specific viruses in otitis media and may contribute to the design of better treatment and prevention strategies for otitis media.

MATERIALS AND METHODS

Recruitment of the Study Cohort

Between November 2007 and May 2009 children between 6 and 36 months of age were recruited for a study investigating the immunology and microbiology of children with recurrent acute otitis media (the GROMIT Study). Cases were defined as children with a history of at least three episodes of acute otitis media requiring the insertion of ventilation tubes. Children requiring the insertion of ventilation tubes for persistent middle ear effusion without a history of acute otitis media were not included. Children undergoing general surgery (predominantly orthopedics, strabismus, circumcision, cryptorchidism, hypospadias repair) and with no history of any form of otitis media, were recruited as healthy controls. At the time of surgery, children were in good health with no evidence of fever or viral infection. Children with diagnosed immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities, and chromosomal or genetic syndromes were excluded. Data on ear disease history 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 before inclusion in the study and the collection of samples.

Sample Collection and Processing

Nasopharyngeal swabs were collected while the child was under general anesthesia for the insertion of ventilation tubes (recurrent acute otitis media group) or minor noninfection related surgical procedures (healthy controls). A sterile flexible cotton-wool 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), placed on ice and transported to the laboratory within 4 hr. Samples were vortexed vigorously for 1 min, the broth was divided equally over two vials and stored at −80 C.
One aliquot was used for the assessment of bacterial pathogens using standard culture methods and the second aliquot was used for the extraction of viral nucleic acid. An anterior–inferior myringotomy incision was made for the collection of middle ear effusion with a sterile Leukotrap (Pall Corporation, Port Washington, NY). The sterile tubing system was flushed with 1 ml of sterile saline to recover all the middle ear effusion. The sample was placed on ice and transported to the laboratory where samples were vortexed vigorously for 1 min. One aliquot of the sample was stored in an equal volume of sterile STGGB at −80°C until use for detection of bacterial pathogens using molecular methods. The second aliquot was used for the extraction of viral nucleic acid.

**Multiplex RT PCR**

The multiplex RT PCR assays have been previously described [Chidlow et al., 2009]. Briefly, nucleic acid was extracted from a 200 μl volume of sample using a modified liquid sample extraction protocol on an automated extraction instrument (Xtractorgene, CAS1820, Corbett, Brisbane, Australia). Standardized amounts of equine herpesvirus and MS2 RNA coliphage were added to the lysis buffer to monitor the efficiency of sample extraction, the removal of PCR inhibitors and the cDNA process. Negative controls were included in the extraction process after every five clinical samples and treated as samples for the completion of the assay. Nucleic acid (8 μl) was added to two multiplex PCR mixes (12 μl) containing 27 and 7 primer pairs. Following 20 amplification cycles, liquid handling robots were used to transfer 1 μl of a 1:10 dilution of that product to several RT PCR (20 μl) mixes containing 2–3 primer pairs. RT PCRs (40 cycles) were conducted in Rotor-Gene 6000 instruments (Corbett, Brisbane, Australia). The multiplex RT PCR assay detects adenovirus types B–E, bocavirus, coronavirus types 229E, OC43, HKU1, and NL63, influenza virus types A–C, parainfluenza virus types 1–4, polyomavirus types K1 and WU, and RSV types A and B. Separate semi-nested PCR assays were performed to detect enterovirus, HRV and human metapneumovirus and PCR products were further characterized by DNA sequencing. Additional characterization of the HRV types utilized primers directed against the VP1 and VP4/2 regions of the genome [Coiras et al., 2004; Ledford et al., 2004; McIntyre et al., 2010].

**Detection of Streptococcus pneumoniae, Nontypeable Haemophilus influenzae, and Moraxella catarrhalis in Nasopharyngeal Swabs and Middle Ear Effusion Samples**

Nasopharyngeal swab samples were examined for the presence of Streptococcus pneumoniae, nontypeable Haemophilus influenzae (NTHi), and Moraxella catarrhalis using standard culture methods as described [Watson et al., 2006]. All presumptive NTHi isolates were further tested by 16SrDNA colony PCR to distinguish true NTHi from nonhemolytic Haemophilus haemolyticus [Murphy et al., 2007]. From the stored middle ear effusion samples, genomic DNA was isolated using the Wizard SV gDNA extraction kit (Promega, Alexandria, Australia) and a pneumococcal lysis buffer. PCR for detection of S. pneumoniae (pneumolysin, ply, Genbank accession number: AAK75991.1; and autolysin A, lytA: NC_005833.1), H. influenzae (Haemophilus protein D, hpd: AAX87718.1), and M. catarrhalis (outer membrane protein, copB: L12346.1) was conducted on gDNA prepared from all middle ear effusion [Wiertsema et al., 2011].

**Statistical Analyses**

Host and environmental risk factors were compared between children with and without recurrent acute otitis media using Mann–Whitney analyses for continuous variables and Pearson chi-square analyses (P-value asymptotic significant two-sided) for categorical variables. The difference in viral detection rates between children with and without recurrent acute otitis media were evaluated using Pearson chi-square analyses. Binary logistic regression was performed to investigate the correlation of viral detection with a history of recurrent acute otitis media correcting for age, gender, day-care attendance, year, and season of sample collection. To investigate the co-detection of viruses with other viruses or any of the three main otitis media bacterial pathogens NTHi, S. pneumoniae or M. catarrhalis in the nasopharynx, chi-square analyses were used. The IBM SPSS Statistics 19 for Windows software package was used for all statistical analyses and P < 0.05 was considered to be statistically significant.

**RESULTS**

**Study Cohort**

In this study 180 children with a history of at least three episodes of acute otitis media in the first 3 years of life and requiring the insertion of ventilation tubes were enrolled. Children in this group were found to have a severe otitis media phenotype, with 84 children (46.7%) having had 8 or more acute otitis media episodes before enrolment. The healthy control group of 66 children had no history of ear disease. The mean age of children with a history of recurrent acute otitis media was 20.9 (7.3–36.0) months and of controls was 18.9 (7.1–35) months (P = 0.12). In the recurrent acute otitis media group, 60.6% of children were male compared with 72.7% in the control group (P = 0.08). There was no difference between the groups in having siblings, with 71.3% of children with recurrent acute otitis media and 73.4% of healthy controls having siblings (P = 0.7). Of the children with a history of recurrent acute otitis media, 60.6% attended day-care ≥4 hr a week compared with 29.7%
of the controls ($P < 0.001$). In the control group significantly more nasopharyngeal samples were collected in winter (52%) compared with the recurrent acute otitis media group (16%).

**Nucleic Acid of Respiratory Viruses Was Detected Commonly in Nasopharyngeal Swabs of Children With and Without a History of Recurrent Acute Otitis Media**

In the children with a history of recurrent acute otitis media, 170/180 (94.4%) had nucleic acid of one or more viruses detected in the nasopharynx whereas this was 47/66 (71.2%) in the healthy controls ($P < 0.001$). Of the children with recurrent acute otitis media, 76.7% were infected with more than one virus whereas this was 25.8% in healthy children ($P < 0.001$). Chi-square analyses showed that compared with healthy controls, children with a history of recurrent acute otitis media were infected more frequently with HRV (58.3% vs. 42.4%; $P = 0.03$), RSV (27.8% vs. 9.1%; $P = 0.002$), HBoV (52.2% vs. 19.7%; $P < 0.001$), adenovirus (25.0% vs. 6.1%; $P = 0.001$), parainfluenza virus (29.4% vs. 9.1%; $P = 0.001$) and polyomaviruses (36.1% vs. 15.2%; $P = 0.002$) (Table I). Subtypes of HRV (A, B, and C), polyomavirus (KI and WU), parainfluenzavirus (1, 2, 3, 4A, and 4B), RSV (A and B), coronaviruses (HKU1, NL63, OC43, and 229E), and influenza virus (A, B, and C) were also determined. The significant difference in detection rates of HRV between children with and without a history of recurrent acute otitis media was due to a difference in infection with HRV A ($P = 0.001$). HRV A accounted for the main portion of HRV types in children with a history of recurrent acute otitis media (64/105, 61.0%), whereas HRV C was the predominant HRV type in healthy controls (19/28, 42.4%). Polymavirus WU was the most common polyomavirus subtype in both children with (44/65, 67.7%) and without recurrent acute otitis media (9/10, 90%), with polyomavirus KI being detected in 25 children with recurrent acute otitis media, but in only 1 healthy control. Parainfluenza virus type 3 was the most common parainfluenza subtype in both children with (47/53, 87.7%) and without a history of recurrent acute otitis media (5/7, 71.4%) and was the type responsible for the significant difference between the two groups ($P = 0.002$). RSV A was the most common RSV subtype in children with recurrent acute otitis media (34/50, 68.0%) whereas within the healthy control group RSV B was more common, however, numbers were low (5/6, 83.3%) (Table I).

Using binary logistic regression adjusting for age, gender, day-care attendance, year, and season of sample collection (odds ratio; 95% confidence interval), carriage of rhinovirus A (2.8; 1.1–6.8), RSV grouped (9.1; 3.1–27.3), RSV A (29.6; 3.6–239.9), HBoV (4.6; 2.1–9.9), adenovirus (4.2; 1.3–13.5), parainfluenza viruses grouped (3.5; 1.2–9.7), parainfluenza virus type 3 (3.4; 1.1–10.5), polyomaviruses (2.5; 1.1–6.0), coronaviruses grouped (4.2; 1.2–14.7), and coronavirus HKU1 (6.4; 1.2–34.9) were significantly associated with a history of recurrent acute otitis media.

**Rhinovirus Was the Predominant Virus Detected in the Middle Ear**

Of the 180 cases, 143 had either unilateral or bilateral middle ear effusion present. In 102 (71.3%) of these children one or more viruses were detected in the middle ear effusion, predominantly HRV, which was detected in the middle ear effusion of 66/143 (46.2%) of these children (Table I). Similar to what was found in the nasopharynx, HRV A was predominant in middle ear effusion (41/66; 62.1%), with HRV C accounting for 26 of the 66 HRV detections (39.4%). HBoV, enteroviruses, and polyomaviruses were detected in middle ear effusion of 12 children each (8.4%) and RSV in middle ear effusion of 11 children. Coronavirus was found in seven, adenovirus and parainfluenzavirus in six, human metapneumovirus in two, and influenza C in one middle ear effusion (Table I).

**Co-Detection of Specific Viruses in the Nasopharynx**

Compared to NPS samples from children with a history of recurrent acute otitis media where no parainfluenza virus was detected (n = 127), parainfluenza positive samples (n = 53) were significantly more often also positive for enterovirus (16/127; 12.6% vs. 15/53; 28.3%; $P = 0.01$), coronavirus (13/127; 10.2% vs. 13/53; 24.5%; $P = 0.01$), human metapneumovirus (2/127; 1.6% vs. 5/53; 9.4%; $P = 0.01$), and RSV (23/127; 18.1% vs. 27/53; 50.9%; $P < 0.001$; Table IIa). In addition to a correlation between adenovirus and parainfluenzavirus in six, human metapneumovirus in two, and influenza C in one middle ear effusion (Table I).

**Co-Detection of Viruses With Bacterial Otopathogens**

Children with a history of recurrent acute otitis media carrying adenovirus (n = 45) carried S. pneumoniae or M. catarrhalis more often than children negative for adenovirus ($P = 0.04$ and $P = 0.005$, respectively; Table IIIa). In addition to a correlation between adenovirus and M. catarrhalis in the nasopharynx, we also
found that carriage of *M. catarrhalis* in the nasopharynx was associated with adenovirus in the middle ear ($P = 0.04$). In healthy children, carriage of *M. catarrhalis* was significantly higher in children positive for parainfluenzavirus in the nasopharynx (5/6; 83.3%) compared to parainfluenzavirus negative samples (23/60; 38.3%; $P = 0.03$; Table IIIb). In healthy children, when HRV was detected ($n = 28$) carriage of *S. pneumoniae* was significantly higher than in HRV negative samples ($n = 38$; $P = 0.03$; Table IIIb). Due to low frequencies, analyses of interactions between bacteria and viruses in the middle ear were limited to NTHi, HRV, and HBoV, which showed that the detection of HRV was significantly associated with infection with NTHi ($P = 0.03$).

**DISCUSSION**

To our knowledge this is the first study comparing the presence of nucleic acid of a wide range of respiratory viruses and bacterial otopathogens, including recently identified viruses such as HBoV, polyomaviruses, human metapneumovirus, and HRV C, in the nasopharynx of children with or without a history of recurrent acute otitis media, demonstrating that viruses were detected frequently in both groups (recurrent acute otitis media 94% and controls 71%). The high detection rates in this study support recent findings from Singleton et al. that showed that 90% of children hospitalized for respiratory infections and 52% of healthy controls had a virus detected in the nasopharynx when using PCR for the detection of seven respiratory viruses, which are similar rates as detected in a case–control study from the Netherlands where samples were investigated for the presence of eight respiratory viruses [van Gageldonk-Lafeber et al., 2005; Singleton et al., 2010]. In contrast, a study by Winther et al. [2007] detected lower virus rates (18.2%) in children without respiratory symptoms despite using PCR techniques, however, PCRs were only performed for the identification of six respiratory viruses.

In the current cross-sectional study, no follow-up data was collected and the high detection rates of viral nucleic acids in asymptomatic subjects might be the result of presymptomatic viral shedding in these children. Several studies have shown however, that with frequent sampling only in 5–20% of cases the

### TABLE I. Detection Rates of Respiratory Viruses in the Nasopharynx and Middle Ear

| Group                           | Healthy rAOM | rAOM | P-value | rAOM |
|---------------------------------|--------------|------|---------|------|
|                                 | NPS          | NPS  | MEE     | MEE  |
| Number samples                  | N = 66       | N = 180 |         | N = 143 |
| Rhinovirus                      | 28 (42.4)    | 105 (58.3) | 0.03     | 66 (46.2) |
| Rhinovirus A                    | 9 (13.6)     | 64 (35.6)   | 0.001     | 41 (28.7) |
| Rhinovirus B                    | 1 (1.5)      | 2 (1.1)      | 0.8       | 0       |
| Rhinovirus C                    | 19 (28.8)    | 39 (21.7)    | 0.3       | 26 (18.2) |
| Bocavirus                       | 13 (19.7)    | 94 (52.2)    | $<0.001$  | 12 (8.4)  |
| Polyomavirus                    | 10 (15.2)    | 65 (36.1)    | 0.002     | 12 (8.4)  |
| PyV WU                          | 9 (13.6)     | 44 (24.4)    | 0.07      | 8 (5.6)   |
| PyV KI                          | 1 (1.5)      | 25 (13.9)    | 0.005     | 4 (2.8)   |
| Parainfluenza virus             | 6 (9.1)      | 53 (29.4)    | 0.001     | 5 (3.5)   |
| PIV 1                           | 1 (1.5)      | 7 (3.9)      | 0.4       | 1 (0.7)   |
| PIV 2                           | 1 (1.5)      | 5 (2.8)      | 0.6       | 1 (0.7)   |
| PIV 3                           | 5 (7.6)      | 47 (26.1)    | 0.002     | 3 (2.1)   |
| PIV 4a                          | 0            | 4 (2.2)      | —         | 0       |
| PIV 4b                          | 0            | 1 (0.6)      | —         | 0       |
| Respiratory syncitial virus     | 6 (9.1)      | 50 (27.8)    | 0.002     | 11 (7.7)  |
| RSV A                           | 1 (1.5)      | 34 (18.9)    | 0.001     | 6 (4.2)   |
| RSV B                           | 5 (7.6)      | 16 (8.9)     | 0.7       | 5 (3.5)   |
| Adenovirus                      | 4 (6.1)      | 45 (25.0)    | 0.001     | 6 (4.2)   |
| Coronavirus                      | 4 (6.1)      | 26 (14.4)    | 0.08      | 7 (4.9)   |
| Cov HKU1                        | 2 (3.0)      | 12 (6.7)     | 0.3       | 6 (4.2)   |
| Cov NL63                        | 2 (3.0)      | 9 (5.0)      | 0.5       | 1 (0.7)   |
| Cov OC43                        | 0            | 8 (4.4)      | —         | 0       |
| Cov 229E                        | 0            | 1 (0.6)      | —         | 0       |
| Enterovirus                     | 5 (7.6)      | 31 (17.2)    | 0.06      | 12 (8.4)  |
| Human metapneumovirus           | 1 (1.5)      | 7 (3.9)      | 0.4       | 2 (1.4)   |
| Influenzavirus                   | 0            | 3 (1.7)      | —         | 1 (0.7)   |
| Influenzavirus A                | 0            | 1 (0.6)      | —         | —       |
| Influenzavirus B                | 0            | 0            | —         | 0       |
| Influenzavirus C                | 0            | 2 (1.1)      | —         | 1 (0.7)   |

Number (%) of children either with recurrent acute otitis media (rAOM) or without a history of rAOM (healthy) with the specific viruses detected in the nasopharynx (NPS) or in middle ear effusion (MEE, children with a history of rAOM only). $P$-value: chi-square analyses comparing detection rates of viruses in the nasopharynx between children with a history of rAOM and healthy children.
same virus is still detectable after 2 weeks [Jartti et al., 2004, 2008; Winther et al., 2006]. This suggests that a respiratory virus may be present in the nasopharynx for a limited amount of time and supports the validity of using PCR to detect “true” respiratory infections. However, next to measuring the presence of viruses, the quantification of viral loads will be important to determine the importance of specific viruses in disease.

In this study the samples were not collected during an acute otitis media episode and therefore a causal relationship between the viral nucleic acid detected in the nasopharynx and otitis media pathogenesis cannot be confirmed. Others have shown that respiratory viruses are isolated from the nasopharynx during an acute otitis media episode in 30–50% of cases using PCR [Nokso-Koivisto et al., 2004; Yano et al., 2009]. Longitudinal studies have also demonstrated that the presence of a virus in the nasopharynx is associated with the subsequent development of otitis media [Winther et al., 2007; Chonnaintree et al., 2008; Alper et al., 2009]. None of these studies had as high detection rates as described in the current study, potentially because a wider range of viruses, such as the more recently discovered HBoV, polymavirus, and human metapneumovirus, was tested for here. In addition, molecular methods were used for the detection of all viruses, whereas other studies used combinations of PCR-, antigen detection-, and culture methods.

Data on the importance of specific viruses in otitis media remain conflicting [Heikkinen and Chonnaintree, 2003; Nokso-Koivisto et al., 2006]. Even though in the current study the classical otitis-prone definition was not used [Howie et al., 1975], children were at the severe end of the otitis media spectrum with a median of seven acute otitis media episodes before the insertion of ventilation tubes. The difference in detection rates of viral nucleic acids in the nasopharynx between this group and a group of children without a history or recurrent acute otitis media was most significant for RSV with an odds ratio of 9.1, supporting a potential role for RSV in otitis media as described by others [Heikkinen et al., 1999; Patel et al., 2007; Yano et al., 2009]. HRV nucleic acid was the type detected most frequently in the middle ear (46%) of children with a history of recurrent acute otitis media which is in accordance with several other studies and suggests HRV may be important in otitis media.

### TABLE IIa. Co-Occurrence of Respiratory Viruses in the Nasopharynx of Children With a History of rAOM.

| rAOM history, N=180 | N | With HBoV | With PyV | With PIV | With RSV | With HAdV | With EV | With CoV | With HMV | With Flu |
|---------------------|---|-----------|---------|---------|---------|----------|--------|---------|---------|--------|
| Rhinovirus positive | 105 | 56 (53.3) | 34 (32.4) | 32 (30.5) | 28 (26.7) | 27 (26.7) | 5 (4.7) | 14 (13.3) | 3 (2.9) | 2 (1.9) |
| Rhinovirus negative | 75 | 38 (50.7) | 31 (41.3) | 21 (28.0) | 22 (29.3) | 17 (22.7) | 26 (34.7) | 12 (16.0) | 4 (5.3) | 1 (1.3) |
| P-value | 0.7 | 0.2 | 0.7 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| Bocavirus positive | 94 | 42 (44.7) | 32 (34.0) | 32 (34.0) | 28 (29.8) | 18 (19.1) | 15 (15.9) | 5 (5.3) | 2 (2.1) |
| Bocavirus negative | 86 | 23 (26.7) | 21 (24.4) | 18 (20.9) | 17 (19.8) | 13 (15.1) | 11 (12.8) | 2 (2.3) | 1 (1.2) |
| P-value | <0.001 | 0.05 | 0.5 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| Adenovirus positive | 115 | 32 (27.8) | 33 (28.7) | 26 (22.6) | 18 (15.7) | 17 (14.8) | 5 (4.3) | 3 (2.6) |
| Adenovirus negative | 127 | 23 (18.1) | 35 (27.6) | 16 (12.6) | 13 (10.2) | 2 (15.7) | 2 (1.6) |
| P-value | <0.001 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| RSV positive | 50 | 10 (20.0) | 12 (24.0) | 12 (24.0) | 5 (10.0) | 2 (4.0) |
| RSV negative | 130 | 35 (26.9) | 19 (14.6) | 14 (10.8) | 2 (1.5) | 1 (0.8) |
| P-value | 0.3 | 0.1 | 0.02 | 0.009 | 0.1 |
| RSV positive | 149 | 19 (12.8) | 5 (3.4) | 3 (2.0) |
| RSV negative | 154 | 2 (7.7) | 2 (7.7) |
| P-value | 0.2 | 0.4 | 0.4 |
| Coronaviruses positive | 26 | 2 (7.7) | 2 (7.7) |
| Coronaviruses negative | 173 | 3 (1.7) |
| P-value | 0.7 |

HBoV, human bocavirus; PyV, polymavirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus; HAdV, human adenovirus; EV, enterovirus; CoV, coronavirus; HMV, human metapneumovirus; Flu, influenza virus.

Number and percentage (between brackets) of samples in which two viruses were detected. P-value (in bold when significant) as determined by Pearson chi-square analyses. Percentage calculated as percentage of the virus positive and virus negative value.
et al., 2004). In addition to the classical HRV species A and B, a third rhinovirus species, HRV C, was identified recently [Arden et al., 2006; Lamson et al., 2006]. It has been suggested that HRV C causes more severe disease, however, debate is ongoing [Arden and Mackay, 2010; Gern, 2010]. HRV A was the predominant HRV type in the nasopharynx and middle ear effusion of children with a history of recurrent acute otitis media described here, whereas HRV C was the most common type in the nasopharynx of healthy children, which does not support a predominant role for HRV C compared to other HRV types in otitis media. In addition, it was demonstrated that in children with a history of recurrent acute otitis media, HRV A accounted for 64/105 (61%) of rhinovirus isolates from the NPS and 41/66 (62.1%) of isolates from the middle ear effusion. HRV C accounted for 39/105 (37.1%) of HRV in the NPS and 26/66 (39.4%) in the middle ear effusion, indicating that neither of the types preferentially resides in the nasopharynx or the middle ear. To our knowledge only one study has investigated HRV types in children with acute otitis media, which found a similar proportion (~50%) of HRV A and HRV C in the nasopharynx and middle ear effusion during acute otitis media [Savolainen-Kopra et al., 2009] supporting our data that there seems to be no difference between the HRV types in their capacity to enter and/or persist in the middle ear.

HBoV was the second most common virus detected in the nasopharynx (52.2%) and middle ear effusion (8.4%) of children with a history of recurrent acute otitis media. Since the discovery of HBoV in 2005 [Allander et al., 2005] debate on the role of HBoV in respiratory disease has been ongoing. Detection rates of HBoV in the nasopharynx of asymptomatic children vary widely from <10% [Kesebir et al., 2006; Brieu et al., 2008; Garcia-Garcia et al., 2008; von Linstow et al., 2008] to two studies describing an infection rate of 40% [Longtin et al., 2008; Martin et al., 2010]. The findings in these latter studies which included otitis-prone children were similar to our observations for children with a history of recurrent acute otitis media. In the current study the detection rate of HBoV nucleic acid in the middle ear effusion was relatively low compared to the detection rate of HBoV in the nasopharynx, which is in agreement with findings from others [Ruohola et al., 2006; Beder et al., 2009; Rezes et al., 2009]. Additional viruses discovered recently, the polyomaviruses KI (KIPyV) and WU (WUPyV) [Allander et al., 2007; Gaynor et al., 2007] were detected in the nasopharynx of 15.2% of healthy controls and 36.1% of children with a history or recurrent acute otitis media. These rates are high compared with other studies where WUPyV and KIPyV is detected in 1–6% of respiratory tract secretions collected during acute respiratory disease.
### TABLE IIIa. Co-Occurrence of Respiratory Viruses With Three Main Bacterial Otitis Media Pathogens S. pneumoniae (Pnc), Nontypeable H. influenzae (NTHi), and M. catarrhalis (Mc) in the Nasopharynx of Children With a History of rAOM

| Virus                      | Total, N | With Pnc, N (%) | With NTHi, N (%) | With Mc, N (%) |
|----------------------------|----------|-----------------|------------------|--------------|
| Rhinovirus positive        | 105      | 40 (38.1)       | 56 (53.3)        | 48 (45.7)    |
| Rhinovirus negative        | 75       | 33 (44.0)       | 44 (58.7)        | 28 (37.3)    |
| Bocavirus positive         | 94       | 37 (39.4)       | 54 (57.4)        | 46 (48.9)    |
| Bocavirus negative         | 86       | 36 (41.2)       | 46 (53.5)        | 30 (34.9)    |
| Polyomavirus positive      | 65       | 25 (38.5)       | 40 (61.5)        | 32 (49.2)    |
| Polyomavirus negative      | 115      | 48 (41.7)       | 60 (52.2)        | 44 (38.3)    |
| Parainfluenzavirus positive| 53       | 20 (37.7)       | 30 (56.6)        | 17 (32.1)    |
| Parainfluenzavirus negative| 127      | 53 (41.7)       | 70 (55.1)        | 59 (46.5)    |
| Respiratory syncytial virus positive | 50 | 19 (38.0) | 29 (58.0) | 22 (44)     |
| Respiratory syncytial virus negative | 130 | 54 (41.5) | 71 (54.6) | 54 (41.5) |
| Adenovirus positive        | 45       | 24 (53.3)       | 24 (53.3)        | 27 (60.0)    |
| Adenovirus negative        | 155      | 49 (36.3)       | 76 (56.3)        | 49 (36.3)    |
| Coronavirus positive       | 26       | 13 (50.0)       | 17 (65.4)        | 10 (38.5)    |
| Coronavirus negative       | 154      | 60 (39.0)       | 83 (53.9)        | 66 (42.9)    |
| Enterovirus positive       | 31       | 13 (40.0)       | 17 (54.8)        | 10 (32.3)    |
| Enterovirus negative       | 149      | 60 (41.9)       | 85 (55.7)        | 66 (44.3)    |
| Human metapneumovirus positive | 7     | 0 (0)           | 3 (42.9)         | 2 (28.6)     |
| Human metapneumovirus negative | 173 | 73 (42.2) | 97 (56.1) | 74 (42.8) |

Number and percentage (between brackets) of samples in which both a virus and bacteria was detected. *P*-value as determined using chi-square analyses. Percentage calculated as percentage of the virus positive and virus negative value.

### TABLE IIIb. Co-Occurrence of Respiratory Viruses With Three Main Bacterial Otitis Media Pathogens S. pneumoniae (Pnc), Nontypeable H. influenzae (NTHi), and M. catarrhalis (Mc) in the Nasopharynx of Healthy Children With No History of rAOM

| Virus                      | Total, N | With Pnc, N (%) | With NTHi, N (%) | With Mc, N (%) |
|----------------------------|----------|-----------------|------------------|--------------|
| Rhinovirus positive        | 28       | 11 (39.3)       | 8 (12.1)         | 12 (42.9)    |
| Rhinovirus negative        | 38       | 6 (15.8)        | 5 (13.2)         | 16 (42.1)    |
| Bocavirus positive         | 13       | 6 (46.2)        | 1 (7.7)          | 6 (46.2)     |
| Bocavirus negative         | 53       | 11 (20.8)       | 12 (22.6)        | 22 (41.5)    |
| Polyomavirus positive      | 10       | 5 (50.0)        | 1 (10.0)         | 2 (20.0)     |
| Polyomavirus negative      | 56       | 12 (21.4)       | 12 (21.4)        | 26 (46.4)    |
| Parainfluenzavirus positive| 6        | 3 (50.0)        | 1 (16.7)         | 5 (83.3)     |
| Parainfluenzavirus negative| 60       | 14 (23.3)       | 12 (20.0)        | 23 (38.3)    |
| Respiratory syncytial virus positive | 6 | 3 (50.0) | 2 (33.3) | 3 (50.0)     |
| Respiratory syncytial virus negative | 60 | 14 (23.3) | 11 (18.3) | 25 (41.7) |
| Adenovirus positive        | 4        | 2 (50.0)        | 1 (25.0)         | 5 (50.0)     |
| Adenovirus negative        | 62       | 15 (24.2)       | 12 (19.4)        | 26 (41.9)    |
| Coronavirus positive       | 4        | 1 (25.0)        | 1 (25.0)         | 3 (75.0)     |
| Coronavirus negative       | 62       | 16 (25.8)       | 12 (19.4)        | 25 (40.3)    |
| Enterovirus positive       | 5        | 2 (40.0)        | 1 (20.0)         | 3 (60.0)     |
| Enterovirus negative       | 61       | 15 (24.6)       | 12 (19.7)        | 25 (41.0)    |
| Human metapneumovirus positive | 1 | 0 (0)          | 0 (0)            | 1 (100)      |
| Human metapneumovirus negative | 64 | 17 (26.6) | 13 (20.3) | 27 (41.5) |

Number and percentage (between brackets) of samples in which both a virus and bacteria was detected. *P*-value as determined using chi-square analyses. Percentage calculated as percentage of the virus positive and virus negative value.
M. catarrhalis was observed. A similar positive association between respiratory viruses in nasopharyngeal samples from 7 (3.0%) and 38 (16.4%) of 232 children with respiratory tract infections [Teramoto et al., 2011]. In addition, the seroprevalence of KIPyV and WUPyV in adults is 55% and 69%, respectively, suggesting that exposure to these viruses, probably in childhood, is very common [Kean et al., 2009]. The fact that in the current study nucleic acid from HBoV, KIPyV, and WUPyV was detected frequently in asymptomatic controls and not commonly in middle ear effusion samples, may suggest their role in otitis media is limited, but further studies are required.

In the current study RSV was associated most frequently with the presence of other viruses in the nasopharynx, which may suggest that RSV facilitates subsequent infections with other viruses. Conversely, a virus that is detected on its own and not associated with other viruses, for example, HRV or adenovirus, may be more important in disease pathogenesis. In nasopharyngeal samples from children with a history of recurrent acute otitis media, a positive association of adenovirus with S. pneumoniae and M. catarrhalis was observed. A similar positive association between adenovirus and M. catarrhalis was found in children in a semi-arid zone of Western Australia, however, a negative association between adenovirus and S. pneumoniae was described in this cohort when using adjusted statistical models [Jacoby et al., 2007; Moore et al., 2010]. A chinchilla model showing adenovirus does not promote otitis media due to M. catarrhalis or S. pneumoniae does not support results described in the current article [Bakaletz et al., 1995; Tong et al., 2000]. In the middle ear, a positive association between HRV and NTHi was observed which was not noted in the nasopharynx, which may suggest that interactions between pathogens are dependent on the local environment. These conflicting results highlight the complexity of pathogen interactions. The interpretation of data from animal models, different human populations, sampling environments, and various statistical models are challenging, further emphasizing that more studies are necessary.

The data described in this article show an extremely high detection rate of nucleic acids of a wide range of respiratory viruses in nasopharyngeal samples from children with and without a history of recurrent acute otitis media. The etiological role of these viruses in recurrent acute otitis media remains uncertain and to establish a causal link with clinical symptoms is challenging, however, anti-viral therapies may be beneficial in future treatment and prevention strategies for acute otitis media.

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