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Detection of Rhinovirus, Respiratory Syncytial Virus, and Coronavirus Infections in Acute Otitis Media by Reverse Transcriptase Polymerase Chain Reaction

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ABSTRACT. *Objective.* **To determine the frequencies of human rhinovirus (HRV), respiratory syncytial virus (RSV), and coronavirus (HCV) infection in children with acute otitis media (AOM).**

Methods. **Middle ear fluids (MEF) collected by tympanocentesis and nasopharyngeal aspirates (NPA) at the time of the AOM diagnosis were examined by reverse transcriptase polymerase chain reaction for HRV, RSV, and HCV RNA.**

Patients. **Ninety-two children aged 3 months to 7 years during a 1-year period.**

Results. **Virus RNA was detected in a total of 69 children (75%) and in 44 MEF samples (48%) and 57 NPA samples (62%) at the time of AOM diagnosis. HRV RNA was detected in both MEF and NPA in 18 (20%), in MEF alone in 4 (4%), and in NPA alone in 10 (11%). RSV was detected in both MEF and NPA in 12 (13%), in MEF alone in 5 (5%), and in NPA alone in 9 (10%). HCV RNA was detected in both MEF and NPA in 5 (5%), in MEF alone in 2 (2%), and in NPA alone in 9 (10%). Dual viral infections were detected in 5% of children. HRV and RSV were detected simultaneously in 2 MEF samples and in 2 NPA samples; RSV and HCV were detected in 1 NPA sample. Bacterial pathogens were detected in 56 (62%) MEF from 91 children. Viral RNA was detected in 20 (57%) MEF of 35 bacteria-negative and in 25 (45%) of 56 bacteria-positive MEF samples. No important differences in the risk of treatment failure, relapse, or occurrence of late secretory otitis media were noted between children with virus-positive and virus-negative MEF aspirates.**

Conclusion. **These findings highlight the importance of common respiratory viruses, particularly HRV and RSV, in predisposing to and causing AOM in young children.** *Pediatrics* **1998;102:291–295;** ***RT-PCR, acute otitis media, rhinovirus, respiratory syncytial virus, coronavirus.***

ABBREVIATIONS. AOM, acute otitis media; RSV, respiratory syncytial virus; HRV, human rhinovirus; RT-PCR, reverse transcriptase polymerase chain reaction; HCV human coronavirus; NPA, nasopharyngeal aspirates; MEF, middle ear fluids; PBS, phosphate-buffered saline.

Several studies have established the association between respiratory virus infections and acute otitis media (AOM) in children.[1–7] The most frequently associated virus in young children has been respiratory syncytial virus (RSV), which has been detected in more than 50% of AOM episodes during outbreak periods.[7] Human rhinovirus (HRV) is of particular interest for AOM in children because it has been reported to be associated with antibiotic failure in mixed/combined bacterial-viral otitis media more often than other respiratory viruses.[1,8] The proportion of AOM associated with HRV has been determined primarily on the basis of HRV isolation in cell culture. However, reverse transcriptase polymerase chain reaction (RT-PCR) has improved the sensitivity of HRV detection in samples collected from persons with acute respiratory illness.[9–13] The relative lack of data on certain respiratory viruses, particularly human coronavirus (HCV), is attributable mainly to the lack of practical diagnostic tests. RT-PCR has also become a useful diagnostic method for HCV infections.[11–13]

Consequently, the purpose of the present study was to use RT-PCR for determining the frequencies of infection by these common respiratory viruses in

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children with AOM. Both middle ear fluid (MEF) samples and nasopharyngeal aspirates (NPA) from children with AOM were tested for HRV, RSV, and HCV RNA. In addition, the virologic findings were compared with bacteriologic and clinical data to assess the impact of viral infection on the outcome of AOM.

MATERIALS AND METHODS

Children

As previously described, 135 children were enrolled in a study of AOM in the Department of Otolaryngology at Helsinki University Central Hospital during March, 1990, to December, 1992.[14] Samples of both MEF and NPA were available from 92 of these children for the present study. A case of AOM was defined as a child having pneumatic otoscopic findings indicative of MEF behind an inflamed tympanic membrane and at least one of the following symptoms of acute infection: otalgia, tugging or rubbing of the ear, rectal or axillary temperature >38.0°C, irritability, restless sleep, acute gastrointestinal symptoms (vomiting or diarrhea), or other simultaneous respiratory infection. Patients without acute symptoms or signs and those with secretory otitis media, tympanostomy tubes, spontaneous perforation of the tympanic membrane, or antibiotic treatment within 1 week before enrollment were excluded from the study.

All enrolled children were treated with oral antibiotics after sample collections. The clinical outcome was evaluated by the same otolaryngologist (J.J.) at scheduled follow-up visits 2 weeks and 6 months after enrollment, as well as at 4-week intervals in between as clinically indicated. Treatment failure was defined as the persistence of pretreatment signs and symptoms at the 2-week follow-up visit. Recurrence was defined as the return of pretreatment signs and symptoms within 7 to 30 days of treatment after initial improvement. Written informed consent was obtained from the parents of all children before enrollment.

Sample Collection and Processing

MEF and NPA samples were obtained at each child's initial visit. The external ear canal was mechanically cleaned and the tympanic membrane was anesthetized with 90% liquefied phenol. A paracentesis lancet was inserted through the anteroinferior part of the tympanic membrane, and MEF was aspirated with an electric suction device into a sterile glass suction tip. Thereafter, the secretion was rinsed out into a polypropylene microtube with 0.5 mL of phosphate-buffered saline (PBS). NPA samples were obtained with a suction catheter inserted through a nostril to a depth of 4 to 8 cm, followed by gentle application of suction with an electric suction device. The secretion was rinsed out as described for MEF. Samples were stored at −70°C until processed for RT-PCR.

If the amount of secretion, MEF or NPA, was sufficient, a cotton-tipped swab was dipped directly into the secretion, and if not, in the PBS-diluted rinse-out. The swabs were immediately placed in modified Stuart transport media (Transpocult, Orion Diagnostica, Espoo, Finland) and transported at 4°C to the bacteriology laboratory. Bacteria were identified using standard laboratory methods. Pneumolysin DNA in MEF samples was detected by PCR as described earlier.[14]

RT-PCR for Viral RNA

RT-PCR for HRV and HCV RNA was done by previously published methods with minor modifications.[13,15] In reverse transcription the virus-specific oligonucleotide primers were: HRV, 5′-GCACTTCTGTTTCCCC-3′; RSV, 5′-TTTGTTATAGGCATATCATTG-3′; HCV 229E, 5′-GGTACTCCTAAGCCTTCTCG-3′; and HCV OC43, 5′-AGGAAGGTCTGCTCCTAATTC-3′. PCR 5′-biotinylated primers were: HRV, 5′-CGGACACCCAAAGTAG-3′; RSV, 5′-TTAACCAGCAAAGTGTTAGA-3′; HCV 229E, 5′-GACTATCAAACAGCATAGCAGC-3′; and HCV OC43, 5′-GCAAAGATGGGGAACTGTGG-3′. Thirty-five cycles of PCR were done using published parameters for HRV and HCV.[13] RSV PCR cycling parameters were 95°C for 2 minutes, 52°C for 1 minute, 72° for 1 minute, and 10-minute extended incubations at 72°C after the last cycle. Rhinovirus type 39 (ATCC, Rockville, MD), a clinical isolate of RSV (provided by Carolyn Crump, University of Virginia, Richmond, VA), HCV 229E (provided by Kathryn V. Holmes, University of Colorado, Denver, CO) and human coronavirus OC43 (ATCC) were used as positive controls, and sterile PBS was used as a negative control in each reaction series.

Unincorporated primers and deoxynucleoside triphosphates were removed from PCR products by select-B spin column (5Prime–3Prime, Boulder, CO). Oligonucleotide probes (HRV, 5′-GCATTCAGGGGCCGGAG-3′; enterovirus, 5′-GGCCGCCAACGCAGCC-3′; RSV, 5′-ACCTGCATTAACACTAAATTC-3′; HCV OC43, 5′-TATTGGGGCTCCTCTTCTG-3′; and HCV 229E, 5′-ACAACACCTGCACTTCCAAA-3′) were labeled at the 3′ end with digoxigenin d-UTP.[12] RSV primer sequences were directed to the F protein mRNA[16,17] at nucleotide positions 796 to 816 (RT-primer), 575 to 594 (PCR primer), and 719 to 739 (RSV probe).

The amplified products were detected using microplate hybridization as previously described.[13] All PCR products from samples positive for a picornavirus were hybridized subsequently with probe specific for enterovirus. PCR products from stock coxsackievirus B1, echovirus 1, and poliovirus 1, prepared in the laboratory, were used as positive controls in this assay.

Data Analysis

Frequencies of proportions between groups were compared by Fisher's exact test.

RESULTS

Patients

The age range of the 92 children (52% boys) was from 3 months to 6 years and 6 months (median, 30 months); 37 children (40%) were under 2 years of age. Most of the AOM cases presented between the months of November and April. The average number of previous AOM episodes was five and the overall frequency of otitis-proneness (defined as ≥3 episodes/6 months) was 25% for the children (Table 1). Seventy children (76%) presented with 1 day or less of earache. Rhinitis was present in 96% and cough in 70% of the children at enrollment, but prolonged respiratory symptoms (>7 days) were present in a minority of children (rhinorrhea in 23%, cough in 13%). Six of the 92 children had MEF sam-

TABLE 1. Demographic and Clinical Data of Children With Acute Otitis Media According to Virologic Findings in the Middle Ear Fluid

Parameter	HRV (n = 22)*	RSV (n = 17)	HCV (n = 7)*	Negative (n = 48)
Age				
<2 Years	9 (41%)	9 (53%)	2 (29%)	17 (35%)
≥2 Years	13 (59%)	8 (47%)	5 (71%)	31 (65%)
Number of earlier AOM episodes (mean)	5	4	5	5
Otitis proneness (≥3 otitis/ 6 months)	5 (23%)	4 (24%)	2 (29%)	12 (25%)
Duration of earache prior to study				
Not described	0	0	1 (13%)	1 (2%)
<6 H	0	3 (18%)	2 (29%)	6 (13%)
6–24 H	14 (64%)	11 (64%)	2 (29%)	32 (67%)
>24 H	8 (36%)	3 (18%)	2 (29%)	9 (18%)
Rhinorrhea >7 Days	6 (27%)	5 (29%)	2 (29%)	8 (17%)
Cough >7 Days	3 (14%)	2 (12%)	1 (14%)	6 (13%)

Abbreviations: HRV, human rhinovirus; RSV, respiratory syncytial virus; HCV, human coronavirus; AOM, acute otitis media.
* Two middle ear fluid samples were positive for both HRV and HCV.

TABLE 2. Detection of Viruses by RT-PCR in Middle Ear Fluid and Nasopharyngeal Aspirates From 92 Children With Acute Otitis Media

Virus	Middle Ear Fluid* *n* (%)	Nasopharyngeal Aspirate† *n* (%)	Children *n* (%)
HRV	22 (24%)	28 (30%)	32 (35%)
RSV	17 (18%)	21 (23%)	26 (28%)
HCV	7 (8%)	14 (15%)	16 (17%)
Total positive	44 (48%)	57 (62%)	69 (75%)

Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; HRV, human rhinovirus; RSV, respiratory syncytial virus; HCV, human coronavirus.
* Two middle ear samples had both HRV and RSV RNA.
† Two nasopharyngeal aspirates had both HRV and RSV RNA, and one had both RSV and HCV RNA.

ples available from both ears. In all these 6 pairs of samples, the viral and bacterial findings were similar in both ears. Therefore, we describe only one MEF result per child.

Virus Infections

Viral RNA was detected by RT-PCR in a total of 69 children (75%), in 44 MEF samples (48%), and in 57 NPA samples (62%) at the time of AOM diagnosis (Table 2). Picornavirus RNA was detected in both MEF and NPA in 18 (20%), in MEF alone in 4 (4%), and in NPA alone in 10 (11%) children with AOM. Because the assay used is picornavirus-specific,[15] the picornavirus-positive samples were tested with an enterovirus-specific probe. None of the RT-PCR picornavirus products hybridized with the enterovirus probe, indicating that they were derived from HRV. Overall, HRV infection was detected in 32 children (35%).

RSV was detected in both MEF and NPA in 12 (13%), in MEF alone in 5 (5%), and in NPA alone in 9 (10%). Overall, RSV infection was detected in 26 children (28%) (Table 2). HCV RNA was detected in both MEF and NPA in 5 (5%), in MEF alone in 2 (2%), and in NPA alone in 9 (10%), so that HCV infection was detected in 16 children (17%). Dual viral infections were detected in 5% of children. Simultaneous HRV and RSV were detected in 2 MEF and in 2 NPA samples; RSV and HCV were detected in 1 NPA sample.

Clinical Correlations

Infection by each of the three viruses was observed across the age range studied. The majority of HRV and most HCV infections were found in children older than 2 years, whereas RSV infections occurred at similar proportions in those older and younger than 2 years (Table 1). The mean number of previous AOM episodes and frequency of otitis-proneness were comparable in the virus-positive and virus-negative groups. There were no differences between virus-positive and virus-negative children with respect to duration of earache before diagnosis (Table 1) or in associated respiratory symptoms. Prolonged rhinorrhea was reported in 30% of virus-positive children before enrollment, compared with 17% of virus-negative children ($P = .29$). Cough was present in 69% of the virus-negative and in 70% of virus-positive children at presentation, but prolonged cough (>7 days) was uncommon in all groups.

Viral and Bacterial Coinfection

Bacterial pathogens were detected in 56 (62%) of 91 MEF samples. *Streptococcus pneumoniae* was found by isolation and/or by pneumolysin PCR in 33%, *Haemophilus influenzae* in 19%, *Branhamella catarrhalis* in 9%, and *Streptococcus pyogenes* in 1% (Table 3). Viral RNA was detected in 20 (57%) of 35 bacteria-negative and in 25 (45%) of 56 bacteria-positive MEF samples ($P = .28$). In 15 MEF samples (16%), neither virus RNA nor bacteria were detected.

Viral RNA was detected in 12 (63%) of 19 NPA samples that were culture-negative for recognized bacterial pathogens and in 48 (69%) of 70 NPA samples that were culture-positive for bacteria (data not shown). In 12 cases (13%) culture was negative both in MEF and NPA samples for bacterial pathogens; 5 of these children had HRV in MEF, 4 had RSV in MEF, and 1 had HCV in MEF.

Outcomes

Treatment failure was seen in 4 (9%) children positive for viral RNA in MEF and in 3 (6%) virus-negative children (Table 4). A recurrence was seen in 5 (11%) children positive for viral RNA in MEF and in 10 (21%) virus-negative children. Among 22 children with HRV RNA detected in MEF, 4 (18%) experienced treatment failure or recurrence. This proportion did not differ from that observed in those with RSV or HCV RNA in MEF (5/24, 21%) or in those without detectable viral RNA in MEF (13/48, 27%).

At the 6-month follow-up, secretory otitis media

TABLE 3. Comparison of Viral RT-PCR and Bacterial Culture Findings in Middle Ear Fluids From 91 Children With Acute Otitis Media

Virus	*Streptococcus pneumoniae**	*Haemophilus influenzae*	*Branhamella catarrhalis*	*Streptococcus pyogenes*	Negative for Bacterial Pathogens	Total
HRV	4	3	4	0	11	22
RSV	3	4	1	0	8	16†
HCV	2	4	0	0	1	7
Negative	21	6	3	1	15	46
Total	30	17	8	1	35	91

Abbreviations: RT-PCR, reverse transcriptase polymerase chain reaction; HRV, human rhinovirus; RSV, respiratory syncytial virus; HCV, human coronavirus.
* *Streptococcus pneumoniae* detected in MEF by isolation and/or by pneumolysin PCR.
† One middle ear fluid was RT-PCR-positive for RSV but not cultured for bacteria. Two MEF samples were positive for both HRV and RSV RNA, but bacterial culture-negative.

TABLE 4. Clinical Outcomes Related to Presence of Viral RNA or Bacteria in Middle Ear Fluid Samples of 91 Children With Acute Otitis Media

Outcome	MEF Findings			
	Virus + Bacteria − $n = 20$	Virus + Bacteria + $n = 25$	Virus − Bacteria + $n = 31$	Virus − Bacteria − $n = 15$
Failure*	2 (8%)	2 (8%)	2 (6%)	1 (7%)
Recurrence†	2 (8%)	3 (12%)	6 (20%)	4 (30%)
SOM within 6 months	6 (24%)	5 (20%)	3 (10%)	3 (20%)

Abbreviations: SOM, secretory otitis media; MEF, middle ear fluid.
* Failure was defined as the persistence of pretreatment signs and symptoms at the 2-week follow-up visit.
† Recurrence was defined as return of signs and symptoms within 7 to 30 days of the treatment after initial improvement.

(glue ear) had developed in 20 children (21%). Of these 14 (70%) had viral infections documented at the time of initial enrollment (3 in NPA only). The risk of developing secretory otitis media was comparable in those with documented viral infection at enrollment (14/69, 20%) and those without (6/23, 26%) ($P = .77$). Based on viral RNA presence in MEF samples, the risk did not differ significantly among those with HRV (5/23, 23%), RSV (4/17, 24%), or HCV (2/7, 29%) infection. In 5 children with late secretory otitis media, HRV was detected initially in both MEF and NPA; in 4 children RSV was detected initially in both MEF and NPA; and in 2 children HCV was detected in both samples. In 1 child HRV was present only in NPA and the bacterial culture was negative in MEF; in 2 children HCV was detected in NPA and the bacterial cultures were negative; in 3 children bacterial culture (2 *S pneumoniae*, 1 *S pyogenes*) was positive in MEF but virus was PCR-negative; and in 3 children both bacterial culture and virus PCR were negative.

DISCUSSION

In the present study we found that common respiratory viruses, specifically HRV, RSV, and HCV, were detectable by RT-PCR in 48% of MEF samples and in 75% of children with AOM. These results confirm and extend earlier studies in which respiratory viruses have been implicated as potential agents of AOM.[1,4,18] HRV was the viral pathogen most commonly detected in this study, and we found HRV by RT-PCR in 24% of MEF samples and evidence of HRV infection in 35% of children with AOM. One previous study using virus isolation found HRV in 8% of MEF from children with AOM.[1] Little information is available about the role of HCV in AOM primarily because of the lack of practical diagnostic methods. We found HCV in 8% of MEF samples and overall in 17% of children with AOM. To our knowledge, this is the first report of HCV association with AOM. One limitation of our study was its restriction to HRV, RSV, and HCV and lack of data regarding other viruses. Infections by other respiratory viruses likely occurred, particularly in the 16% of cases in which neither bacteria nor viruses were detected. Nevertheless, our results indicate that most episodes of AOM in children are associated with common respiratory viruses.

Although RT-PCR is more sensitive than other methods for HRV and HCV detection, its role in enhancing the sensitivity of RSV detection has been unclear. A previous study has shown that RT-PCR for RSV increases the detection rate minimally compared with antigen tests in NPA,[19] but RT-PCR may be more sensitive for MEF samples.[7] We detected RSV RNA in MEF from 18% of children with AOM. In a previous study the highest RSV detection rate in the MEF samples in children with AOM was 53% during an epidemic situation.[7] Other studies using culture and antigen detection for RSV have found frequencies of 5%[2] to 15%[4] in MEFs, which are comparable to our findings. Although it is not clear that RT-PCR adds sensitivity to detection of RSV compared with conventional antigen assays and isolation in cell culture,[19,20] the use of a panel of RT-PCR primer pairs is applicable to the detection of several different viral RNAs and to small volume samples, such as middle ear effusions.

The effect of concurrent viral infection on the clinical course of AOM is incompletely characterized. We did not find major differences in the clinical course of AOM when children with and without viral RNA in MEF samples were compared. Two previous studies[2,8] reported that the presence of cultivable HRV together with bacteria in the MEF was associated with a higher bacteriologic failure rate in AOM compared with the presence of other respiratory viruses (RSV, adenoviruses, influenza, and parainfluenza viruses). In these earlier studies the follow-up visit and routine tympanocentesis were scheduled 2 to 6 days after starting antibiotic therapy, and the numbers of patients with specific viral etiologies were small. We did not find a specific association between HRV infection and poor outcome, but our study differed in having the first follow-up visit after 2 weeks and in using clinical monitoring (ie, no routine follow-up tympanocentesis). Consequently, our results are not directly comparable with these earlier trials. Other studies[4,21] in which the role of specific virus type was not clear reported that the presence of virus in the MEF was associated with worsening of the clinical course of bacterial AOM. However, any comparison of the outcomes of mixed viral-bacterial infections with bacteria alone depends heavily on the sensitivity of the methods used to determine the presence of viral infection. Our high frequency of viral infection suggests that earlier studies have underestimated the contribution of certain respiratory viruses to AOM in children.

Because RT-PCR is an extremely sensitive method, it is possible that a positive RT-PCR in the MEF may represent residual RNA rather than active infection. The question remains whether active viral replication occurs in the middle ear or whether the viral products detected in the MEF derive from replication in the nasopharynx. Another question emerging from studies using RT-PCR is whether prolonged subclinical infections exist. Although 12% of asymptomatic children and 4% of asymptomatic adults were found positive for picornavirus in nasal wash-

ings by PCR,[11] we are unaware of comparable studies concerning RSV or HCV. The finding that HRV (18/32, 56%) and RSV (12/26, 46%) were detected simultaneously in MEF and NPA in approximately half of the infections caused by these viruses suggests that the presence of viral RNA in the MEF represents active viral infection in the upper respiratory tract, rather than prolonged persistence of viral RNA. This is also supported by the results of earlier studies using virus isolation from MEF. However, the available data do not permit conclusions as to the site of viral replication that is the origin of the RNA detected in the MEF.

It is important to emphasize that bacterial pathogens were frequently absent (38% of MEF samples) and that viral RNA was detected in 57% of bacteria-negative MEF samples. These findings suggest that antimicrobial therapy may be unnecessary in a significant proportion of AOM cases, but further studies will be needed to help practitioners determine which patients do not need antibiotic treatment. Furthermore, these results underscore the need for interventions to prevent and treat respiratory viral infections. Of note, prevention of particular virus infections by influenza vaccine[22] and RSV immunoglobulin[23] has been associated with reduced frequencies of AOM during the winter months. It remains to be determined whether early therapy with selective antiviral agents could reduce the risk of AOM in children with acute upper respiratory illnesses attributable to specific viral infections.

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