Polymerase chain reaction–based detection of rhinovirus, respiratory syncytial virus, and coronavirus in otitis media with effusion

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Objectives: To study the association of human rhinovirus (HRV), respiratory syncytial virus (RSV), and human coronavirus infections in children aged 6 months to 12 years with otitis media with effusion (OME). To determine how long HRV RNA can be detected after HRV infection.

Methods: Middle ear effusion (MEE) samples collected at the time of tympanostomy tube placement from 100 children with OME were examined. Viral RNA was detected by reverse-transcriptase polymerase chain reaction. For HRV the results were compared with virus isolation in cell culture. In vitro studies of the persistence of HRV infectivity and RNA were conducted by combining ~10^5 median cell culture infectious doses of HRV with pooled MEE at 37°C and assaying serial samples for 12 weeks.

Results: Virus RNA was detected in 30 children. HRV was detected by reverse-transcriptase polymerase chain reaction in 19 children with OME and by virus isolation in 5 children. RSV RNA was found in 8 and HCV in 3 children with OME. No dual viral infection was found. Bacterial pathogens were isolated from 35 MEE samples and were associated with viral RNA in 11 cases, most often with HRV (9 cases). Under in vitro conditions, HRV culture positivity declined rapidly (<2 days), but RNA was detectable for up to 8 weeks.

Conclusions: These results suggest that virus infection, particularly HRV infection, either alone or concurrent with bacteria, is present in a larger percentage of children with OME than previously suspected. It remains to be determined how often the presence of viral RNA in MEE represents persistent RNA, ongoing viral replication, or recurrent infection. (J Pediatr 1998;133:390-4)

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Otitis media with effusion is a common childhood disease without a completely clarified etiology. OME has been suggested to be a continuum of acute otitis media.[1] However, despite the routine use of antibiotics in AOM, the incidence of OME in the United States has been estimated to have increased 250% between 1975 and 1990,[2] although epidemiologic data supporting this conclusion are weak. Young children experience frequent upper respiratory tract illnesses, up to 8 to 12 episodes per year; the commonly associated viruses are human rhinoviruses, respiratory syncytial virus, and human coronaviruses. Although up to 80% of middle ear effusions have been shown to contain bacterial DNA by polymerase chain reaction–based amplification techniques,[3,4] antibiotic treatment infrequently affects the course of OME.[5,6] Several earlier studies indicate that viruses may play a role in the pathogenesis of OME.[7,8] Previous diagnostic tools to detect HRV in MEEs have been limited to standard culture methods, and there has not been a practical diagnostic method for HCV infection.

AOM	Acute otitis media
HCV	Human coronavirus
HRV	Human rhinovirus
MEE	Middle ear effusion
OME	Otitis media with effusion
PBS	Phosphate-buffered saline
RSV	Respiratory syncytial virus
RT-PCR	Reverse-transcriptase polymerase chain reaction

In this study we examined MEEs from children with OME undergoing tympanostomy for the presence of HRV by reverse-transcriptase PCR testing and virus culture, and for RSV and HCV by RT-PCR. To study the potential persistence of HRV RNA in MEE, we also serially assayed HRV infectivity and RNA detectability in pooled MEE samples to which HRV had been added in vitro.

METHODS

Children

The study population comprised 100 children, aged 6 months to 12 years, who were admitted during 1993 to 1994 for scheduled operation (tympanostomy and/or adenoidectomy) to the Depart-

ment of Otolaryngology, Helsinki University Central Hospital, because of persistent OME. The criteria for diagnosis of OME included the presence of effusion behind an intact eardrum as determined by pneumatic otoscopy for at least 1 month, subsequently confirmed by tympanostomy, without signs and symptoms of acute infection. In children with bilateral involvement, MEE was collected only from the right ear. Children who had purulent MEE or those who had received antimicrobial therapy or experienced upper respiratory tract infections within 1 week before the study were not included. The duration of OME was based in each case on the clinical follow-up data recorded by the patient's physicians. The patients were enrolled in the study after informed consent had been obtained from the parents. All received general anesthesia for the procedure.

Table I. Clinical data in children with OME and associated viral infection*

	Virus RNA–positive			
	HRV	RSV	HCV	Total
Gender				
Boys (n = 67)	13	7	2	22 (31%)
Girls (n = 31)	4	1	1	6 (19%)
Age (y)				
<2 (n = 44)	7	6	1	14 (32%)
≥2 (n = 54)	10	2	2	14 (26%)
Effusion type				
Mucoid (n = 52)	8	6	2	16 (27%)
Mucopurulent (n = 42)	9	2	1	12 (28%)
Serous (n = 4)	0	0	0	0
Duration of effusion (mo)				
≤2 (n = 28)	5	1	0	6 (21%)
3-4 (n = 38)	3	5	1	9 (24%)
5-6 (n = 22)	10	2	1	13 (58%)
≥6 (n = 10)	1	0	1	2 (20%)
Number with prior tympanostomy tubes (n = 25)	4	3	1	8 (32%)
Number with prior adenoidectomy (n = 26)	6	3	2	11 (42%)
Number with ≥4 episodes of AOM in 6 mo (n = 34)	5	4	1	10 (29%)

*Clinical data from 2 children are missing; both were positive for HRV as determined by RT-PCR.

Sample Processing

All MEE samples were obtained during tympanostomy under the control of an operating microscope by aspiration with an electric suction device into a Tym-Tap collector (Juhn Tym-Tap; Xomed Inc, Jacksonville, Fla). A cotton-tipped swab was dipped directly into the aspirate. The swabs were immediately placed in modified Stuart transport media (Transpocult; Orion Diagnostica, Espoo, Finland) and transported at +4°C to the microbiology laboratory. The residual sample was rinsed out into a polypropylene microtube with 0.5 mL of phosphate-buffered saline and stored at –70°C until processed. MEE samples were cultured for bacteria, and the isolates were identified by standard methods.[9] Nineteen MEE samples positive for HRV as determined by RT-PCR were inoculated onto monolayers of human embryonic fibroblastic cells (WI-38 stain) and incubated as previously described.[10]

RT-PCR

The RT-PCR protocols and primer sequences for HRV and HCV were based on published methods with minor modifications.[11-16] Primers for RSV were directed to the F protein messenger RNA[17] in the following nucleotide positions: 796-816 for the reverse transcription primer, 575-594 for the upstream biotinylated primer, and 719-739 for the internal probe. Briefly, RNA was extracted by using matrix affinity chromatography (QIAamp blood kit; QIAGEN, Chatsworth, Calif). Reverse transcription was carried out in 21 μL volume with virus-specific oligonucleotide primers (HRV, 5′-GCACTTCTGTTICCCC-3′; RSV, 5′-TTTGTTATAGGCATATCATTG-3′; HCV 229E, 5′GGTACTCCTAAGCCTTCTCG-3′; HCV OC43 5′-AGGAAGGTCTGCTCCTAATTC-3′). After reverse transcription (MMLV-RT; Gibco BRL, Gaithersburg, Md) reactions, the samples were heated to inactivate the transcriptase at 95°C for 10 minutes and mixed with 24 μL of 5′-biotinylated primer (HRV, 5′- CGGACACCCAAAGTAG-3′; RSV, 5′-TTAACCAGCAAAGTGTTAGA-3′; HCV 229E, 5′-GACTATCAAACAGCATAGCAGC-3′; HCV OC 43, 5′-GCAAAGATGGGGAACTGTGG-3′) and 5.8 μL of PCR buffer K (Invitrogen, San Diego, Calif) to adjust the final concentrations of primers and $MgCl_2$ to 0.8 μmol/L and 2.7 mmol/L, respectively, and the final pH to 9.0. While tubes were held at 80°C, 1.25 U of Taq Polymerase (Applied Biosystems, Foster City, Calif) diluted in 5 μL of water was added, and 35 cycles of PCR of denaturation (1 minute at 95°C for HRV; 2 minutes at 95°C for HCV and RSV), annealing (1.5 minutes at 48.2°C for HRV; 1 minute at 60°C for HCV; 1 minute at 52°C for RSV), DNA synthesis (1 minute at 72°C), and extension 10 minutes at 72°C. Rhinovirus type 39 (ATCC, Rockville, Md), RSV, HCV 229E (provided by Kathryn V. Holmes, University of Colorado), and OC43 (ATCC) were used as positive controls; and sterile PBS was used as negative control in each reaction series. Unincorporated primers and deoxyribonucloside triphosphates were removed from PCR products by SELECT-B spin column (5Prime-3Prime, Boulder, Colo). Oligonucleotide probes (HRV 5′-GCATTCAGGGCCGGAG-3′; enterovirus 5′GGCCGCCAACGCAGCC-3′; RSV 5′ACCTGCATTAACACTAAATTC-3′; HCV OC43 5′TATTGGGGCTCCTCTTCTG-3′; HCV 229E 5′ACAACACCTGCACTTCCAAA-3′) were labeled at the 3′ end with digoxigenin deoxy-uridine-5′-triphosphate by using 3′ nucleotidyltransferase, according to protocol from the manufacturer (Boehringer Mannheim, Germany).

Table II. Detection of HRV, RSV, and HCV from middle ear fluid in children with OME

	Middle ear fluid		
	Virus alone	Combined with pathogenic bacteria*	Total
Rhinovirus (%)	8 (42%)	9 (58%)	19†
RSV (%)	7 (88%)	1 (12%)	8
Coronavirus (%)	2 (67%)	1 (33%)	3
Total	17	11	30†

**Streptococcus pneumoniae, Haemophilus influenzae, Branhamella catarrhalis.*
†Two samples were RT-PCR–positive for HRV, but no bacteriologic data were available.

Care to minimize the chance of cross-contamination and contamination by previously amplified DNA included physical segregation of sample preparation and PCR preparation under 2 separate laminar flow hoods and the execution of PCR product analysis in a different laboratory, as well as use of positive-displacement pipettes and disposable gloves, gowns, and masks. Virus-spiked MEEs and dilutions of virus stocks were used as positive controls, and samples testing negative for HRV, HCV, and RSV, as well as buffer-only reactions, were used as negative controls.

Detection of Amplification Products

The amplified products were detected as previously described by using microtiter plate hybridization.[16,17] All PCR products from samples positive for a picornavirus were hybridized subsequently with a probe specific for enterovirus. PCR products from stock coxsackie virus B1, echovirus 1, and poliovirus 1 (prepared in the laboratory) were used as positive controls in this assay.

In Vitro Studies

Pooled HRV RNA-negative MEE from children (volume, ~0.5 mL per experiment) or an equivalent volume of PBS was inoculated with approximately 10^5 median tissue culture infectious doses of HRV type 39. After mixing, a 50 μL aliquot was removed and combined with 450 μL of PBS (dilution, 1:10) for titration in HeLa cells, and the remainder was frozen at –70°C for later assay for RNA by RT-PCR (described above). The inoculated samples were then incubated at 37°C. Uninoculated samples of MEE and PBS were processed in parallel. In the first experiment serial aliquots (50 μL) were removed on days 1, 2, 4, 7, 21, 28, and 35 and combined with 450 μL PBS for testing as described above. In the second experiment, which used only MEE samples, aliquots were removed at baseline and then in weeks 1, 2, 4, 8, and 12.

RESULTS

Study Population

Clinical and demographic data were available for 98 of 100 MEE samples. These 98 samples were collected from 67 boys and 31 girls, ranging in age from 6 months to 12 years, with a median age of 26 months (Table I). Most of the effusions were either mucoid or mucopurulent. The proportion of effusions positive for HRV, RSV, and HCV did not differ by effusion type (Table I). The duration of MEE varied from 1 to 12 months (median, 3 months) before sampling, and in 90% of the cases MEE had persisted for 2 months or longer.

Microbiologic Findings

HRV RNA was detected by RT-PCR in 19 of 100 MEEs. Cultures of 19 MEEs positive by RT-PCR had recoverable HRV in 5 children (26%). Because the RT-PCR assay used is picornavirus-specific,[15] the positive samples were tested with an enterovirus-specific probe. None of the RT-PCR picornavirus-positive products hybridized with the enterovirus probe, indicating that all positive samples represented HRV. RSV was detected in 8 of 100 and HCV was detected in 3 of 100 MEE samples. Overall, 30% of the children were positive for 1 of these 3 viruses. No dual viral infections were found.

Bacterial pathogens were isolated from 35 MEEs and were associated with virus RNA in 11 cases, of which 9 were HRV (Table II). HRV positivity tended to be associated more frequently with presence of bacterial pathogens (58%) compared with RSV (12%) or HCV (33%; $P = .09$, HRV vs RSV, 2-tailed Fisher's exact test).

Among virus-positive MEEs, HRV predominated in those 2 years of age or older (10 of 14, 71%), whereas RSV (6 of 14, 43%) and HRV (7 of 14, 50%) were almost equally prominent in children younger than 2 years of age. Over 50% of HRV-positive samples occurred in children who had 5- to 6-month histories of effusion (Table I). The proportions of children who had prior tympanostomy tubes, prior adenoidectomy, or frequent bouts of acute otitis were comparable across the virus-positive groups and also among the 30 virus-positive children (27%, 37%, and 33%, respectively) compared with the 70 virus-negative children (24%, 21%, and 34%, respectively). HRV was detected throughout the year except during July and August, whereas 50% of RSV-positive samples occurred in November.

In Vitro Studies

After inoculation with a high concentration of infectious HRV, culture positivity was lost by 2 days in the pooled MEE sample but gradually decreased over 7 days in PBS (Table III). In contrast, detection of HRV RNA by RT-PCR persisted for 35 days in the MEE sample but was lost by day 21 in PBS. A second experiment confirmed that HRV RNA detectability lasted up to 8 weeks but was lost by 12 weeks of in vitro incubation.

DISCUSSION

In this study we detected HRV, RSV, or HCV RNA in 30% of the MEE samples from children with OME. The use of RT-PCR for amplification of HRV RNA increased the detection rate nearly 4-fold

Table III. Persistence of rhinovirus type 39 infectivity and RNA detectability in middle ear effusion fluid under in vitro conditions

	Viral titer/RNA detectability on day								
	0 (baseline)	1	2	4	7	14	21	28	35
Experiment 1									
MEE + HRV	4.0/P	0.5/P	N/P	N/P	N/P	N/P	N/P	N/P	N/P
PBS + HRV	3.5/P	3.0/P	1.5/P	1.0/P	N/P	N/P	N/N	N/N	-/-
MEE alone	N/N	N/N	N/N	N/N	N/N	N/N	N/N	N/N	N/N
	Viral titer/RNA detectability in week								
	0 (baseline)	1	2	4	8	12			
Experiment 2									
MEE + HRV	3.25/P	N/P	–/P	–/P	–/P	–/N			
MEE alone	N/N	N/N	–/N	–/N	–/N	–/N			

Results of viral titers are expressed in $\log_{10}$ median cell culture infective dose per milliliter.
P, Positive; *N,* negative or no growth; –, not tested.

compared with culture alone (19% vs 5% of samples). This corresponds to an earlier study in which RT-PCR increased the frequency of picornavirus detection in nasal samples up to 4-fold compared with culture of samples obtained from adult patients with asthma.[18] Our rate of culture positivity is similar to that of another study in which HRV was recovered in 8% of subacute or chronic cases of OME.[7] In one previous study RSV was found in MEEs of children with OME by RT-PCR with a frequency of 10%,[8] which is similar to our 8% rate of RSV detection. In another study[19] RSV RNA was detected by RT-PCR in middle ear exudates collected by myringotomy in 21 of 34 children with otitis media during and after an RSV outbreak.

It is uncertain how often detection of respiratory viral RNA represents persistent infection, residual noninfectious RNA, or intercurrent infections in these children. Recently, it has been shown that children prone to OME tend to have more frequent discrete episodes of effusions rather than increased overall duration of episodes.[20] Unfortunately, our study did not incorporate the simultaneous collection of nasopharyngeal samples to examine for presence of infection at that level of the respiratory tract. Of note, PCR-based amplification techniques can detect the presence of bacterial DNA in a significant percentage of bacterially culture-negative MEEs in children.[3,4] In the middle ear cavities of experimentally infected chinchillas, purified bacterial DNA and DNA from intact but nonviable bacteria do not persist in MEE, although antibiotic-treated bacteria can persist in some viable state for weeks.[21] In contrast, it is not known how long viral RNA can remain in MEE or mucosa without being degraded. In separate studies we found that HRV RNA is detectable by RT-PCR only up to 3 days longer than by culture in nasal washings of experimentally infected adults (Pitkäranta A, unpublished observations, 1997). However, the duration of HRV persistence in the nasopharynx may differ from that in the middle ear, and the duration of shedding may be shorter in adults than in children, in part because of differences in immune competence. In order to address indirectly the question of HRV persistence in MEE, we collected HRV-negative MEEs and added large amounts of infectious HRV to determine the durability of infectivity and RNA detectability by our assays. Although cultivable virus was lost rapidly in MEE, RNA detectability persisted for 35 days in one experiment and for 8 weeks in another. These in vitro studies raise the question of whether RNA detected in MEEs sometimes represents residual viral RNA in the thick effusion. However, our results (5/19 culture-positive MEEs) and previous findings of HRV culture-positive MEE samples[6] clearly indicate active HRV infection in some children. Whether this represents chronic or recurrent infection remains to be determined. Direct demonstration of viral replication in situ or amelioration of the condition by specific antiviral therapy would provide evidence for productive viral infection in the middle ear.

We found direct evidence of co-infection by virus, particularly HRV, and bacteria in 11% of our patients. Although the mechanisms are not defined, earlier studies showed that co-infection with rhinovirus and bacteria was an indicator for failure of resolution of AOM during antibiotic treatment.[22,23] These findings support the concept that respiratory viruses, either alone or in combination with bacteria, are commonly associated with OME. Viral infection can cause obstruction of eustachian tube and abnormalities of middle ear pressure during both experimentally induced[24] and natural HRV infections.[25] This may in turn cause impaired drainage of the middle ear and represent one possible mechanism contributing to the development of OME. Changes in local immune responses in the middle ear may be another contributory factor, particularly when secondary bacterial infection occurs,[11] although the mechanism is not clear.

This cross-sectional study examined a heterogeneous population and did not incorporate sequential sampling from the time of development of AOM, so that

conclusions about pathogenesis and causation are not possible. However, our results indicate that viruses, particularly HRV, either alone or concurrently with bacteria may be present in a greater percentage of children with OME than previously suspected. Effective methods for preventing and/or treating these viral infections could have a significant impact on this important clinical problem.

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REFERENCES

1. Giebink GS. Otitis media update: pathogenesis and treatment. Ann Otol Rhinol Laryngol Suppl 1992;155:21-3.
2. Adam D, Ehrlich GD. Otitis media: prospects for management. Royal Turnbridge Wells, Kent (UK: Wells Medical Limited; 1996. Chapel Place International Clinical Practice Series.
3. Post JC, Preston RA, Aul JJ, Larkins-Pettigrew M, Rydquist-White J, Anderson KW, et al. Molecular analysis of bacterial pathogens in otitis media with effusion. JAMA 1995;273:1598-604.
4. Hendolin PH, Markkanen A, Ylikoski J, Wahlfors JJ. Use of multiplex PCR for simultaneous detection of four bacterial species in middle ear effusions. J Clin Microbiol 1997;35:2854-8.
5. Giebink GS, Batalden PB, Le CT, Lassman FM, Buran DJ, Seltz AE. A controlled trial comparing three treatments for chronic otitis media with effusion. Pediatr Infect Dis J 1990;9:33-40.
6. Cantekin EI, McGuire TW, Griffith TL. Antimicrobial therapy for otitis media with effusion ('secretory' otitis media) [see comments]. JAMA 1991;266:3309-17.
7. Arola M, Ziegler T, Puhakka H, Lehtonen OP, Ruuskanen O. Rhinovirus in otitis media with effusion. Ann Otol Rhinol Laryngol 1990;99:451-3.
8. Shaw CB, Obermyer N, Wetmore SJ, Spirou GA, Farr RW. Incidence of adenovirus and respiratory syncytial virus in chronic otitis media with effusion using the polymerase chain reaction. Otolaryngol Head Neck Surg 1995;113:234-41.
9. D'Amato RF. Bacteriology. In: Balows A, Hausler WJ, Herrmann KL, Isenberg HD, Shadomy HJ, editors. Manual of clinical microbiology. Washington (DC): American Society for Microbiology; 1991. p. 209-578.
10. Arruda E, Crump CE, Rollins BS, Ohlin A, Hayden FG. Comparative susceptibilities of human embryonic fibroblasts and HeLa cells for isolation of human rhinoviruses. J Clin Microbiol 1996;34: 1277-9.
11. Storgaard M, Larsen K, Blegvad S, Nodgaard H, Ovesen T, Andersen PL, et al. Interleukin-8 and chemotactic activity of middle ear effusions. J Infect Dis 1997;175:474-7.
12. Kamahora T, Soe LH, Lai MM. Sequence analysis of nucleocapsid gene and leader RNA of human coronavirus OC43. Virus Res 1989;12:1-9.
13. Myint S, Harmsen D, Raabe T, Siddell SG. Characterization of a nucleic acid probe for the diagnosis of human coronavirus 229E infections. J Med Virol 1990;31:165-72.
14. Arruda E, Hayden FG. Detection of human rhinovirus RNA in nasal washings by PCR. Mol Cell Probes 1993;7: 373-9.
15. Myint S, Johnston S, Sanderson G, Simpson H. Evaluation of nested polymerase chain methods for the detection of human coronaviruses 229E and OC43. Mol Cell Probes 1994;8:357-64.
16. Pitkäranta A, Arruda E, Malmberg H, Hayden FG. Detection of rhinovirus in sinus brushings of patients with acute community-acquired sinusitis by reverse transcription-PCR. J Clin Microbiol 1997;35:1791-3.
17. Collins PL, Huang YT, Wertz GW. Nucleotide sequence of the gene encoding the fusion (F) glycoprotein of human respiratory syncytial virus. Proc Natl Acad Sci USA 1984;81:7683-7.
18. Nicholson KG, Kent J, Ireland DC. Respiratory viruses and exacerbations of asthma in adults. BMJ 1993;307:982-6.
19. Okamoto Y, Kudo K, Shirotori K, Nakazawa M, Ito E, Togawa K, et al. Detection of genomic sequences of respiratory syncytial virus in otitis media with effusion in children. Ann Otol Rhinol Laryngol Suppl 1992;157:7-10.
20. Hogan SC, Stratford KJ, Moore DR. Duration and recurrence of otitis media with effusion in children from birth to 3 years: prospective study using monthly otoscopy and tympanometry. BMJ 1997;314:350-3.
21. Post JC, Aul JJ, White GJ, Wadowsky RM, Zavoral T, Tabari R, et al. PCR-based detection of bacterial DNA after antimicrobial treatment is indicative of persistent, viable bacteria in the chinchilla model of otitis media. Am J Otolaryngol 1996;17:106-11.
22. Arola M, Ziegler T, Ruuskanen O. Respiratory virus infection as a cause of prolonged symptoms in acute otitis media. J Pediatr 1990;116:697-701.
23. Sung BS, Chonmaitree T, Broemeling LD, et al. Association of rhinovirus infection with poor bacteriologic outcome of bacterial-viral otitis media. Clin Infect Dis 1993;17:38-42.
24. Buchman CA, Doyle WJ, Skoner D, Fireman P, Gwaltney JM. Otologic manifestations of experimental rhinovirus infection. Laryngoscope 1994;104:1295-9.
25. Elkhatieb A, Hipskind G, Woerner D, Hayden FG. Middle ear abnormalities during natural rhinovirus colds in adults. J Infect Dis 1993;168:618-21.