

Detection of Rhinovirus in Sinus Brushings of Patients with Acute Community-Acquired Sinusitis by Reverse Transcription-PCR

ANNE PITKÄRANTA,^{1,2} EURICO ARRUDA,^{1,3} HENRIK MALMBERG,² AND FREDERICK G. HAYDEN^{1*}

*Departments of Internal Medicine and Pathology, University of Virginia, Charlottesville, Virginia 22908¹;
Department of Otolaryngology, Helsinki University Hospital, FIN-00290 Helsinki, Finland²; and
Faculty of Medicine, University of Sao Paulo, Ribeirão Preto, SP, Brazil 14049-900³*

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Of 20 adults with acute community-acquired sinusitis (ACAS), rhinovirus was detected in specimens from 10 (50%) patients, including maxillary aspirates from 8 (40%) patients and nasal swabs from 9 (45%) patients, by reverse transcription-PCR (RT-PCR). Human coronavirus was detected by RT-PCR in nasal swabs from 3 of 20 patients but in no sinus secretions. These findings suggest that rhinovirus is an important cause of ACAS and that viral invasion of the sinus cavity itself may be a common event during the disease.

Most cases of acute community-acquired bacterial sinusitis are believed to be secondary to a preceding viral upper-respiratory-tract infection. This view has been supported by earlier studies in which respiratory viruses were isolated from sinus aspirates of patients with acute community-acquired sinusitis (ACAS) (5, 9). More recently, sinus abnormalities were detected by magnetic resonance imaging in volunteers with experimental rhinovirus colds (17) and by computed tomography in patients with natural rhinovirus colds (7). An important question in understanding the pathogenesis of viral sinusitis is whether direct viral invasion of the sinus cavity or inflammatory events in adjacent areas in the nose, such as the osteomeatal complex, are responsible for the abnormalities in the sinus cavity.

The agents most frequently implicated as causes of viral upper-respiratory-tract infections are human rhinoviruses (HRV) and human coronaviruses (HCV) (6, 15). The advent of reverse transcription-PCR (RT-PCR) has enabled the detection of picornavirus RNA directly in clinical samples with higher levels of sensitivity (2, 8, 11) than viral isolation in cell culture (2, 11). In the present study, the maxillary sinus of adult patients with ACAS was punctured, and specimens obtained with a cytologic brush were tested for HRV RNA and HCV RNA by RT-PCR.

MATERIALS AND METHODS

Patients. Twenty adult patients with diagnoses of ACAS based on clinical findings were studied between May and July 1996 in the Department of Otolaryngology of the University Hospital of Helsinki. All patients had purulent rhinorrhea and nasal obstruction, and most had facial pain. The patients had not been treated with antibiotics, and the duration of their symptoms was less than 2 weeks. Sinus X rays were obtained for 18 of the patients. Ten patients had an air-fluid level in the maxillary sinus, and six of these were culture positive for bacteria (Table 1). Eight patients had mucosal thickening of >8 mm, and two of these had positive bacterial cultures. There were 11 females (ages, 23 to 65 years) and 9 males (ages, 29 to 60 years); the median age was 38 years. Patients for whom frontal sinusitis was suspected were excluded, as were patients with histories of chronic sinusitis, allergic rhinitis, or asthma. Both maxillary antra were punctured in 10 patients, the left antrum was punctured in 6 patients, and the right antrum was punctured in 4 patients.

Specimen collection. Prior to puncture, a nasal swab was obtained from each patient at the area of puncture below the inferior turbinate and was placed in

phosphate-buffered saline (PBS) for viral culture and for RT-PCR. The puncture area was then anesthetized topically with 10% lidocaine and adrenaline. After puncture with a needle, a bronchoscope brush (diameter, 2 mm; length, 11 mm) was passed through the needle into the sinus and rotated. Upon removal the brush was twirled in 0.5 ml of PBS. Samples were stored at -70°C until they were processed for RT-PCR. Samples were also cultured for HRV on monolayers (0.1 ml per monolayer) of human embryonic fibroblasts (WI-38 strain) and HeLa-I cells by previously described methods (3). Following collection of the brush samples, antral lavage with saline was done to obtain samples for bacterial culture. The washings were collected from the nares and inoculated with cotton swabs onto agar transport tubes (Transpocult; Orion Diagnostica, Espoo, Finland), and bacteria were identified by standard laboratory procedures.

All patients were treated with an oral antibiotic (usually amoxicillin) for 10 days and had a follow-up visit after 1 week and once weekly thereafter, if symptoms persisted. Two patients also had effusions in the middle ear and underwent tympanocentesis. Samples of these aspirates were also tested for viral RNA and bacteria.

RT-PCR. (i) HRV RT-PCR. Total RNA was extracted from 100 μl of sample, diluted in an equal volume of PBS, by matrix affinity chromatography (QIAamp blood kit; Qiagen, Chatsworth, Calif.). The RNA was eluted in 200 μl of the kit's elution buffer. HRV reverse transcription was carried out with primer 2 as previously described, except that 10 μl of RNA was used (2). HRV reverse transcription (Moloney murine leukemia virus reverse transcriptase; Gibco BRL, Gaithersburg, Md.) reaction mixtures were heated at 95°C for 10 min to inactivate reverse transcriptase and were mixed with 24 μl of PCR mixture, containing 2 μl of 5'-biotinylated primer 1 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 μM and 2.7 mM, respectively, and to adjust 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 H_2O was added, and 35 cycles of PCR were performed with published parameters (2). Rhinovirus type 39 (American Type Culture Collection, Rockville, Md.) was used as a positive control, and normal nasal washings, as well as sterile PBS, were used as negative controls, in each reaction series.

(ii) HCV RT-PCR. The procedure for HCV RT-PCR was the same as that for HRV RT-PCR, except that pairs of primers for both coronavirus OC43 and coronavirus 229E were used in the same reaction, in a multiplex format, and the parameters for 35 cycles of denaturation, annealing, and DNA synthesis were 2 min at 95°C , 1 min at 60°C , and 1 min at 72°C , respectively. This was followed by a final extension period of 10 min at 72°C . HCV 229E (kindly provided by Kathryn V. Holmes, University of Colorado) and OC43 (American Type Culture Collection) were used as positive controls, and PBS was used as a negative control, in each reaction series.

Precautions to minimize cross-contamination of the samples with PCR product included the use of positive displacement pipettes and separate rooms for sample preparations and amplification analysis. During the various steps of the work, separate pipettes, gloves, and coats were used. Nonincorporated primers and deoxynucleoside triphosphates were removed from PCR products by using a SELECT-B spin column (5 Prime \rightarrow 3 Prime, Boulder, Colo.), and the product was frozen at -20°C until it was used for hybridization. HRV primer 1 (5'-GC ACTTCTGTTTCCCC-3'), HRV primer 2 (5'-CGGACACCCAAAGTAG-3'), HRV oligonucleotide probe (5'-GCATTCAGGGGCCGAG-3'), enterovirus oligonucleotide probe (5'-GGCCGCAACGCAGCC-3'), HCV 229E primer 1 (5'-GGTACTCCTAAGCCTTCTCG-3'), HCV 229E primer 2 (5'-GACTATC AACAGCATAGCAGC-3'), HCV 229E oligonucleotide probe (5'-ACAACA

* Corresponding author. Mailing address: University of Virginia, Health Sciences Center, Box 473, Charlottesville, VA 22908. Phone: (804) 924-5059. Fax: (804) 924-9065.

TABLE 1. Data from 20 adult patients with acute community-acquired maxillary sinusitis^a

| Duration of symptoms (days) | Abnormal X-ray result ^b | Detection ^c of: | | | |
|-----------------------------|------------------------------------|----------------------------|---------|---|---|
| | | Rhinovirus (max/nose), by: | | Coronavirus OC43 ^d (max/nose), by RT-PCR | Bacterium, by culture ^e |
| | | RT-PCR | Culture | | |
| 5 | + | +/+ | +/+ | -/- | Negative |
| 5 | + | -/- | -/- | -/- | Negative |
| 6 | NT | +/+ | +/+ | -/- | <i>Fusobacterium</i> sp. |
| 6 | ++ | -/+ | -/- | -/- | <i>Streptococcus milleri</i> |
| 7 | ++ | -/- | -/- | -/- | <i>Streptococcus pneumoniae</i> |
| 7 | + | -/- | -/- | -/- | Negative |
| 8 | ++ | +/+ | +/+ | -/- | <i>Haemophilus influenzae</i> |
| 9 | + | -/- | -/- | -/+ | <i>S. pneumoniae</i> |
| 10 | + | -/- | -/- | -/- | <i>S. pneumoniae</i> |
| 10 | + | -/- | -/- | -/- | Negative |
| 12 | ++ | +/+ | -/- | -/- | Negative |
| 12 ^f | ++ | -/- | -/- | -/- | <i>Klebsiella pneumoniae</i> |
| 12 ^f | + | -/- | -/- | -/- | Negative |
| 13 | ++ | +/+ | -/- | -/- | Negative |
| 13 | + | +/+ | -/- | -/- | Negative |
| 13 | ++ | +/+ | -/- | -/- | Negative |
| 13 | NT | +/+ | -/- | -/- | <i>H. influenzae</i> |
| 13 | ++ | -/+ | -/- | -/- | <i>S. pneumoniae</i> |
| 13 | ++ | -/- | -/- | -/+ | <i>H. influenzae</i> and <i>S. pneumoniae</i> |
| 13 | ++ | -/- | -/- | -/+ | Negative |

^a Each row of data represents one patient.

^b ++, maxillary effusion; +, mucosal thickening or thick secretions; NT, not tested.

^c max/nose, maxillary sinus sample/nasal sample.

^d All nose and maxillary samples were negative for HCV 229E RT-PCR.

^e Cultures were not quantitative.

^f Middle-ear effusions from these patients were negative by HRV and HCV RT-PCR and bacterial culture.

CCTGCACTTCCAAA-3'), HCV OC43 primer 1 (5'-AGGAAGGTCTGCTCC TAATTC-3'), HCV OC43 primer 2 (5'-GCAAAGATGGGGAAGTGTGG-3'), and HCV OC43 oligonucleotide probe (5'-TATTGGGGCTCCTCTCTG-3') were obtained from Midland Certified Reagents (Midland, Tex.). Oligonucleotide probes were labelled at the 3' end with digoxigenin dUTP by using 3' nucleotidyltransferase, following the protocol from the manufacturer (Boehringer Mannheim Corp., Indianapolis, Ind.). The locations of these primer sequences within the picornavirus and coronavirus sequences have been published previously (2, 10, 12-14).

Detection of amplification products. Microplate hybridization was carried out by published methods with minor modifications (18). Ten microliters of the PCR product was added to streptavidin-coated microplate wells in 40 μ l of binding buffer consisting of 25 mM Tris-HCl (pH 8.0), 125 mM NaCl, 5 mM EDTA, 5 \times Denhardt's solution, and 0.1% Tween 20, and the mixture was shaken (at 150 rpm) for 30 min at room temperature. Double-stranded PCR product was denatured by incubation for 2 min at room temperature with 100 mM NaOH-300 mM NaCl. Wells were washed three times with 200 μ l of washing buffer (20 mM Tris-HCl, 125 mM NaCl, 20 mM MgCl₂, 5% Tween 20), and 50 μ l of hybridization solution was added. The hybridization solution consisted of 1 \times SSC (0.15 M NaCl plus 0.015 M sodium citrate), 1 \times Denhardt's solution, 0.1% sodium dodecyl sulfate, 20 mM EDTA, and 10 pmol of oligonucleotide probe specific for either picornavirus, enterovirus, HCV 229E, or HCV OC43. The plates were incubated at 45°C in a water bath with shaking for 30 min and were washed six times with 0.01 \times SSC-0.3% Tween 20. Following incubation with 5% fetal bovine serum in PBS-0.1% Tween 20 for 30 min and washing with PBS-Tween 20, the detection of hybrids by colorimetric reaction was carried out by published methods (4). The threshold of positivity was defined as the mean of three negative controls plus three times the standard deviation of the mean. All PCR products from samples positive for a picornavirus were hybridized subsequently with probes 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.

RESULTS

Picornavirus RNA. Picornavirus RNA was detected by RT-PCR in the maxillary sinus brushings from 8 of 20 patients with ACAS (40%) and in the nasal swab samples from 9 of 20 patients (45%) (Table 1). Since the assay used is picornavirus specific (2), we tested the picornavirus-positive samples with an

enterovirus-specific probe and found that none of the RT-PCR picornavirus products hybridized with the enterovirus probe. For seven patients (35% of the total), both maxillary sinus and nasal samples were positive for HRV. Overall, HRV was detected in 10 of 20 patients (50%). By culturing of nasal swabs and maxillary brush samples, HRV was detected in both sample types from two patients and in only the maxillary sinus sample from one patient. All HRV culture-positive samples were also RT-PCR positive. The culture-positive cases all presented within 8 days of symptom onset, whereas the majority (five of eight) of those with negative cultures but positive RT-PCR for HRV RNA presented later (Table 1).

HCV RNA. None of the sinus samples were positive for HCV 229E or OC43. Three nasal swab samples were positive for HCV OC43, and two of these patients had positive bacterial cultures (Table 1). All three of the HCV-positive patients were negative for rhinovirus infection by RT-PCR and culture.

Bacterial culture. Bacterial cultures were positive in 10 (50%) of 20 patients. RT-PCR of sinus brushings was positive for HRV RNA in 5 of 10 samples negative for bacteria and in 3 of 10 samples positive for bacteria. Of 10 HRV-infected patients, 5 had positive bacterial cultures of antral washings. In five samples (25% of the cases), both the RT-PCR and bacterial cultures were negative, and in three samples (15% of the cases), both the RT-PCR and bacterial cultures were positive. The two samples from middle-ear effusions were negative by bacterial culture and by RT-PCR for HRV and HCV RNA.

DISCUSSION

In the present study, we found that HRV can be detected by culture in 15% of the brush samples obtained by puncture of the maxillary sinuses in adults with ACAS and can be detected by RT-PCR in 40% of these samples. Our culture results con-

firm earlier studies of ACAS which reported recovery of HRV in maxillary secretions aspirated from 8 and 7% of patients (5, 9). However, the use of RT-PCR enhanced detection of HRV infection, particularly in samples that were obtained during the 2nd week after the onset of illness. These results are consistent with previous studies of persons with acute respiratory illness which found a high prevalence of positive RT-PCR results for picornavirus in association with negative cultures (11). While our findings may have resulted from viral replication in the sinus, they may also have been caused by the presence in the sinus of virions or viral RNA produced by replication elsewhere in the upper-respiratory-tract epithelium and introduced during coughing or sneezing, or potentially even by HRV RNA introduced to the sinus at the time of puncture. Direct demonstration of viral replication *in situ* would provide definitive evidence of productive HRV infection in the sinus mucosa. While not proving direct viral causation for acute sinusitis during the course of HRV infection, the observation that HRV was detected in the sinus in 40% of 20 patients with acute sinusitis and overall in 50% of these patients strongly suggests the importance of HRV infection in predisposing to ACAS.

These samples were also tested for another important cause of upper-respiratory-tract infections, HCV (15). Although the coronavirus OC43 was found in three nasal swab samples, none of the sinus samples were positive for HCV 229E or OC43. The 15% coronavirus infection rate is consistent with the findings of prior studies addressing the contributions of coronaviruses to colds (15). Of note, HCVs typically cause infections during the winter months, and our patients were studied during the late spring and early summer. Overall, HRV or HCV infection was documented in 65% of these cases. Other respiratory viruses were not sought in the context of these studies, in part because of the limited sample volume and because of patient sampling after the end of the influenza and respiratory syncytial virus seasons. However, the findings indicate that a preceding respiratory viral infection is associated with the majority of ACAS cases.

These findings support the concept that viruses predispose to bacterial infection of the maxillary sinuses. Although the bacterial causes of ACAS have been extensively studied, few prospective studies have assessed the role of viruses in the etiology of these infections (5, 9). In a study of adults experimentally infected with HRV, changes in the paranasal sinuses were observed by magnetic resonance imaging in 33% of volunteers (17). In natural colds, sinus abnormalities are observed by computed tomography in over 85% of patients (7). These changes resolved spontaneously after several weeks in most patients. These studies indicate that an upper-respiratory viral infection may induce paranasal sinus effusions or mucosal thickening indistinguishable from that observed in acute bacterial sinusitis. We found direct evidence of coinfection by HRV and bacteria in three (15%) of our patients. Although the mechanisms are not defined, earlier studies found that rhinovirus-bacterium coinfection was an indicator of a poor prognosis of acute otitis media during antibiotic treatment (1, 16). While the same could be true with acute sinusitis as well, the limited number of patients we studied does not allow conclusions in this regard. However, the importance of HRV

infection in causing sinus disease alone and in conjunction with bacteria raises the possibility that early treatment of HRV colds with specific antirhinovirus agents could have an impact on the risk of development of sinusitis.

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