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Original Article

Respiratory viruses in children with cystic fibrosis: viral detection and clinical findings

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Background Viral detection from different respiratory sample types in children with cystic fibrosis (CF) is facilitated by available molecular methods, but optimum sampling strategies have not been identified. In addition, associations between viral detection and respiratory symptoms are not well described.

Objectives Study goals were to compare molecular detection of viruses from concurrent upper airway and sputum samples in children with CF and to describe relative frequency of respiratory viral infections and identify potential clinical associations.

Methods We conducted a 2-year prospective surveillance study in 44 children with CF aged 6–18 years. Upper airway and sputum samples were collected quarterly and during pulmonary exacerbations and tested for respiratory syncytial virus (RSV), influenza viruses, parainfluenza viruses types 1–4, human metapneumovirus, coronaviruses, rhinoviruses, and adenoviruses. Physical exams and symptom surveys were used to identify respiratory signs and symptoms.

Results Upper airway samples were collected at 359 visits; concordance of PCR-based viral detection was examined in a subset of paired upper airway and sputum samples from 21 participants at 92 visits. Rhinovirus was the most commonly detected virus (23·1% overall), and rhinovirus detection was the same for both sample types (21·7% each). Sensitivity and specificity for the detection of rhinovirus in sputum relative to upper airway sampling were 70% and 91·7%, respectively. Respiratory symptoms associated with rhinovirus detection included increased cough, increased nasal congestion, increased sputum production, and wheezing.

Conclusions A relatively high frequency of rhinovirus detection was observed by either upper airway or sputum samples, and clinical findings suggest a significant-associated symptom burden.

Keywords Adenovirus, coronavirus, cystic fibrosis, rhinovirus, sputum.

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Introduction

Prior to the use of molecular detection methods to detect respiratory viruses, detection rates of respiratory viruses in clinical specimens from children with cystic fibrosis (CF) were remarkably low.[1] Viral detection in this patient population was complicated by multiple factors, including tenacious respiratory specimens that inhibited viral growth in cell culture, bacterial and fungal overgrowth contaminating respiratory specimens, and the general problems with cultivation of difficult-to-propagate respiratory viruses such as rhinovirus, coronavirus, and other viral pathogens. The use of potentially insensitive serologic assays further hampered the detection of respiratory viral infections in this patient population. None-the-less, the association of respiratory viral infections with exacerbations of lung disease and overall progression of CF airway disease has been appreciated for a number of years.[1–4]

Molecular diagnostic techniques circumvent the problems associated with viral culture in children with CF and permit the detection of viruses that are extremely difficult or impossible to culture even in optimal settings.[2,5–7] Multiple viruses, including influenza, parainfluenza, adenovirus, and respiratory syncytial virus (RSV), have been clearly associated with pulmonary exacerbations and worsening lung function in children with CF.[2,6,8,9] However, clinical symptoms associated with new respiratory viruses such as coronavirus and human metapneumovirus are not well described, and the symptomatic impact of rhinovirus, the most commonly detected virus in CF patients,[2,6,8,9] is not

clear. Although respiratory viruses have been detected in both upper and lower airway samples, previous studies in patients with CF have not directly compared detection of respiratory viruses in upper airway and sputum samples.

We performed a 2-year prospective cohort study in school-age children with CF to compare respiratory virus detection in upper airway and sputum samples. Real-time polymerase chain reaction (PCR) for the detection of respiratory viruses was performed on respiratory specimens collected during routine clinic visits and during pulmonary exacerbation. Paired upper airway and sputum samples were collected on a subset of participants within the larger cohort study, and the concordance of PCR-based viral detection between sample types was determined. The association between the detection of respiratory viruses and clinical symptoms was also evaluated.

Materials and methods

Study population

Children with the diagnosis of CF between 6 and 18 years of age who attended clinics at Seattle Children's Hospital were eligible for enrollment. Lung transplant recipients were excluded. This study was approved by the Institutional Review Board at Seattle Children's Hospital.

Study design

Participants were enrolled between January 2006 and 2007 and followed prospectively for up to 2 years. Study visits occurred at scheduled quarterly clinic visits and during pulmonary exacerbations. At each visit, history and medications were reviewed to determine occurrences of pulmonary exacerbation, defined as hospitalization or treatment with intravenous antibiotics for respiratory indications. Respiratory system physical findings were recorded, and symptom surveys were completed by participants to record presence/absence of respiratory symptoms at each visit and during the preceding 30 days. Sputum bacterial culture results were also recorded.

A nasal sample and oropharyngeal (OP) swab were collected at each visit. If participants were able to expectorate, sputum was also collected. The nasal sample consisted of either a nasal wash or a deep nasal swab, which was placed into specimen lysis buffer. The subsequently collected OP swab was placed in the nasal wash or lysis buffer, such that the upper airway sample reflected a combined nasal/OP specimen. All samples were stored frozen at −80°C until processed for viral testing.

Respiratory virus detection

Nucleic acids were extracted from each sample, either upper airway or sputum, and amplified with specific primers to detect 11 common respiratory viruses: respiratory syncytial virus, influenza virus types A and B, parainfluenza virus types 1–4, human metapneumovirus, human coronaviruses (subtypes OC43, 229E, NL63 and HKU1), rhinoviruses, and adenoviruses. Specimens were tested by a panel of seven single or multiplex real-time reverse transcription (RT)-PCR and PCR (for adenovirus) for qualitative detection of these respiratory viruses as previously described.[10–14]

The upper airway sample was extracted as previously described.[10] Sputum was diluted 1:1 in Sputolysin (6·5 mm dithiothreitol) and extracted using the QIAamp RNA Mini Kit (Qiagen, Inc., Frederick, MD, USA). Samples were eluted in 200 μl of water. EXO RNA was added to lysis buffers as an extraction and amplification control.[10] Each assay reliably detected 10 viral copies per reaction, providing a sensitivity of 1000 copies/ml (10 μl of specimen added per reaction).

RT-PCR mixes were premade by adding the correct amount of specific primers and probes to seven reaction tubes, which were dried and stored frozen for up to 3 months. For amplification, 25 μl of a one-step RT-PCR master mix (AgPath; Applied Biosystems, Foster City, CA, USA) and 10 μl of extracted sample were added to a seven-well set. Results were reported as positive (amplification plot crossed the threshold at <40 cycles) or negative (amplification plot did not cross the threshold) for each target in each well. Samples negative for respiratory viruses with average EXO threshold cycles >35·5 were unsatisfactory, and extraction and/or amplification were repeated.

A new rhinovirus assay that detected more rhinovirus subtypes than detected by the original assay was introduced during the study.[14] Extracted nucleic acid samples that had been stored at −80°C for up to 42 months were thawed and retrospectively tested by the new assay for the majority of samples that had been studied to date. For purposes of data analysis, we defined a positive rhinovirus result as a positive finding by either assay; for comparison of paired samples, all pairs were judged using the same version of the assay.

Statistical analysis

Frequency of virus detection in upper airway and sputum samples was summarized by counts and proportions. Repeated measures regression models that accounted for repeated observations per participant were used to analyze concordance between paired samples, with a dependent variable that reflected whether results within each sample pair were the same or different. Sensitivity and specificity of sputum samples for virus detection relative to upper airway samples as the reference standard were estimated as proportions, with associated 95% confidence interval (CI) estimates obtained using the sandwich estimator of variance in logistic regression models that accounted for repeated observations per participant. Potential predictors of respiratory virus detection (concurrent respiratory ill-

ness, respiratory signs or symptoms, and bacterial culture results) were examined in logistic regression models that adjusted for age and accounted for repeated observations per participant; estimates of the odds ratio (OR), 95% CI, and *P*-value were obtained for each model. Reported *P*-values were not adjusted for multiple comparisons. Analyses were performed using Stata (Release 10·1; StataCorp, College Station, TX, USA).

Results

Patient demographics

A total of 44 participants were enrolled. The mean age at enrollment was 12·8 years; mean FEV_1 percent predicted was 94·2% (Table 1). An upper airway sample was collected at each of 359 visits during the study period, for an average of 8·2 samples per participant (SD 2·4, range 5–14). Most nasal samples were collected by nasal swab rather than nasal wash because of participant preference.

A paired sputum specimen was collected simultaneously with the upper airway sample from 21 participants who were able to expectorate at one or more visits during the study. This sub-cohort of expectorating patients was similar to the total cohort in terms of demographics, but had lower BMI percentiles and worse lung function at enrollment than observed for the full cohort (Table 1). Paired sputum samples were collected at 98 visits; however, samples from six visits could not be assayed (four because of failure to amplify and two were missing). Thus, paired sputum samples were available from 92 visits (23% of which were characterized by pulmonary exacerbation).

Table 1. Demographic data for full cohort and for the sub-cohort of participants with paired sputum samples collected

	Full cohort, *n* = 44	**Sub-cohort, *n* = 21**
	***n* (%)**	***n* (%)**
Sex		
Male	19 (43·2)	10 (47·6)
Female	25 (56·8)	11 (52·4)
Race/ethnicity		
Caucasian (not Hispanic)	43 (97·7)	20 (95·2)
Hispanic	1 (2·3)	1 (4·8)
Genotype		
Homozygous	29 (65·9)	14 (66·7)
Heterozygous	11 (25·0)	6 (28·6)
Other	4 (9·1)	1 (4·8)
Pancreatic status		
Sufficient	5 (11·4)	1 (4·8)
Insufficient	39 (88·6)	20 (95·2)
	Mean (SD)	Mean (SD)
Age at enrollment (years)*	12·8 (3·0)	13·0 (2·9)
Sweat chloride (mEq/l)**	106·2 (15·4)	105·4 (13·5)
BMI percentile	45·9 (27·1)	38·9 (25·6)
FEV_1 percent predicted***	94·2 (18·2)	83·6 (15·0)
FVC percent predicted***	99·3 (16·0)	90·5 (13·2)

*Age ranged from 6·1 to 17·7 years for the full cohort and 7·5–17·6 years for the sub-cohort.
**Sweat chloride was not required if there were two identifiable mutations consistent with cystic fibrosis; sweat chloride data were available for 40 participants in the full cohort and 18 in the sub-cohort.
***Lung function measures reflect the best result from clinic visits during 12 months prior to screening; spirometry data were missing for one participant in the full cohort (testing not performed because of developmental delay).

Viral detection in upper airway samples

Among the 359 upper airway samples tested by PCR, at least one respiratory virus was detected in 108 samples (30·1%), with two or more respiratory viruses detected in six specimens. The average number of positive upper airway samples per participant was 2·5 (SD, 1·9; range, 0–9). Rhinovirus was the most commonly detected virus, found in 83 samples (23·1%), with results for additional viruses summarized in Table 2. Among the 44 study participants, 41 (93·2%) had at least one positive upper airway sample during the 2-year follow-up. Thirty-nine participants (88·6%) had rhinovirus detected at one or more visits, including 10 participants with rhinovirus detected twice and 11 with rhinovirus detected at three or more visits (up to a total of eight visits). The proportion of visits with rhinovirus detection was highest during the fourth quarter of the year (37·5% versus 14·6%, 23·9%, and 19·0% for first through third quarters, respectively).

Viral detection in paired upper airway and sputum samples

Among the 92 visits with paired sputum samples also collected, rhinoviruses were the most commonly detected virus, with the same detection rate for both sample types (21·7% each, Table 2). For all other virus types, the number of positive samples was low, including 56 of 92 pairs (60·9%) with both sample types negative for all viruses tested. For rhinoviruses, there were 80 concordant pairs (14 positive/positive; 66 negative/negative) and 12 discordant pairs (six upper positive/sputum negative; six upper negative/sputum positive). For other virus types, there were 85 concordant pairs (seven positive/positive; 78 negative/negative) and seven discordant pairs (four upper positive/sputum negative; three upper negative/sputum positive). There was no evidence of a significant difference in the types of discordant pairs for rhinoviruses or for

Table 2. PCR-based virus detection in 359 upper airway samples and in 92 sets of paired upper airway and sputum samples

	Virus detections in upper airway samples (n = 359)	Virus detections in paired upper airway and sputum samples (n = 92)	
	Number* (%)	Upper Airway Number (%)	Sputum Number (%)
Rhinovirus	83 (23·1)	20 (21·7)	20 (21·7)
Adenovirus	6 (1·7)	4 (4·4)	3 (3·3)
Influenza A	1 (0·3)	1 (1·1)	3 (3·3)
Influenza B	3 (0·8)	0 (0)	0 (0)
Parainfluenza 1	3 (0·8)	1 (1·1)	0 (0)
Parainfluenza 2	0 (0·0)	0 (0)	0 (0%)
Parainfluenza 3	7 (1·9)	2 (2·2)	1 (1·1)
Parainfluenza 4	1 (0·3)	0 (0)	0 (0)
Respiratory syncytial virus	2 (0·6)	0 (0)	0 (0)
Coronavirus	3 (0·8)	2 (2·2)	3 (3·3)
Human metapneumovirus	6 (1·7)	1 (1·1)	0 (0)

*108 positive samples accounted for 115 positive results: 102 samples with one virus detected, five samples with two viruses detected, and one sample with three viruses detected. The five samples with two viruses detected all had rhinovirus with another virus, including adenovirus (n = 2), human metapneumovirus (n = 1), parainfluenza 1 (n = 1), and parainfluenza 3 (n = 1). The one sample with three viruses detected had adenovirus, human metapneumovirus, and parainfluenza 3.

other virus types combined (P = 1·0 and P = 0·67, respectively). We estimated the sensitivity and specificity of sputum sampling for rhinovirus detection relative to detection by upper airway sampling; the observed sensitivity was 70·0% (95% CI 48·0, 85·5) and specificity was 91·7% (95% CI 83·7, 95·9). Estimates of sensitivity and specificity were not calculated for other virus types because of their low relative frequency.

Clinical features associated with upper airway rhinovirus detection

As rhinovirus was the most prevalent virus type detected in our study, further analyses examined rhinovirus detection in association with clinical parameters. These analyses were restricted to the subset of 329 upper airway samples with rhinovirus as the only virus detected (n = 78) or with no viruses detected (n = 251); samples with isolation of other virus types (n = 25) or with co-isolation of rhinovirus and another virus type (n = 5) were excluded. Among the 329 visits giving rise to these samples, 130 (39·5%) visits were characterized by respiratory illness requiring antibiotic treatment (intravenous or inhaled antibiotics for pulmonary exacerbation or other antibiotic treatment for respiratory-related illness). Rhinovirus was detected in 35 of 130 respiratory illness visits (26·9%) and in 43 of 199 non-illness visits (21·6%). Findings from a logistic regression model were not significant for an association between respiratory illness and concurrent detection of rhinovirus (OR, 1·4; 95% CI 0·9, 2·1; P = 0·17). When respiratory illnesses requiring antibiotic treatment up to 30 days prior to the study visit were included, the association with the detection of rhinovirus was slightly stronger (OR 1·6; 95% CI 0·95, 2·7; P = 0·07).

Among respiratory signs recorded at each visit, rhinorrhea (OR 2·0; 95% CI 1·2, 3·2; P = 0·004) and abnormal chest exam (OR 2·3; 95% CI 1·2, 4·4; P = 0·01) were positively associated with upper airway detection of rhinovirus. Among concurrent respiratory symptoms collected by participant self-report, increased cough, increased nasal congestion, increased sputum production, and wheezing were significant predictors of rhinovirus detection (Table 3). Increased cough and increased sputum production were most predictive of rhinovirus detection among symptoms

Table 3. Upper airway rhinovirus detection in association with concurrent respiratory symptoms

	Detection of rhinovirus*		
Respiratory symptoms reported at visit	OR	95% CI	*P*-value
Decreased appetite	0·8	0·3, 2·3	0·71
Muscle aches	1·0	0·2, 4·4	0·97
Headache	0·9	0·3, 2·4	0·76
Increased nasal congestion	2·2	1·2, 4·1	0·009
Sore throat	1·4	0·4, 5·1	0·61
Increased cough	2·2	1·4, 3·3	0·0003
Increased sputum production	2·2	1·2, 4·0	0·01
Change in sputum appearance	2·3	0·9, 6·1	0·09
Wheezing	2·8	1·1, 6·8	0·02
Shortness of breath	1·7	0·7, 4·0	0·26
Increased chest congestion	1·9	1·0, 3·8	0·06
Chest pain	0·7	0·4, 1·4	0·36
Increased fatigue	0·8	0·3, 2·2	0·68

CI, confidence interval; OR, odds ratio.
*Each row shows results for a separate logistic regression model. The odds ratio associated with each symptom was estimated relative to a baseline category of symptom not present. Each model included adjustment for age and accounted for repeated observations per participant. Models were restricted to the 329 visits with rhinovirus only detected or with no viruses detected; respiratory symptom data were available for 326 of these 329 visits. Models were not estimated for fever or chills due to sparse data.

reported for the 30 days preceding the clinic visit, with results similar to those observed for concurrent symptoms.

Bacterial culture results were also evaluated to determine whether concurrent culture positivity for *Pseudomonas aeruginosa* or *Staphylococcus aureus* was associated with upper airway rhinovirus detection. Among 265 visits with concurrent culture data available, *S. aureus* positive cultures were associated with rhinovirus detection (OR, 2·8; 95% CI 1·0, 7·3; $P = 0·04$); however, *P. aeruginosa* positive cultures were not (OR 0·8; 95% CI 0·4, 1·4; $P = 0·36$).

Discussion

The evaluation of respiratory viruses in specimens from children with CF using sensitive and specific molecular methods of detection has only been recently reported. In 2005, Punch *et al.*[8] evaluated RT-PCR detection of seven common respiratory viruses in CF sputum. Olesen *et al.*[9] evaluated PCR detection from either laryngeal aspirates or sputum, but without paired samples from the upper airway. Other studies have evaluated the relative frequency of respiratory viruses in nasal swabs from CF individuals, without evaluating their presence in sputum.[15,16] Unique aspects of the current study include the evaluation of additional novel viruses including human metapneumovirus and diverse strains of coronaviruses, examination of potential associations between viral detection and patient-reported symptoms and clinical signs, and prospective evaluation of paired upper airway and sputum samples from children with CF.

We demonstrated PCR detection of respiratory viruses in upper airway and sputum samples at similar rates, and we did not find that discordant results were associated with sample type. Of note, the detection of these viruses in single specimens demonstrates the increased level of viral detection compared to reports using viral culture.[1] Our results in combination with previous reports demonstrate the usefulness of molecular detection of viruses for children with CF.

Despite the large number of respiratory viruses detectable with our respiratory viral panel, the majority of viruses identified throughout our study in both upper airway and sputum specimens from children with CF were rhinoviruses. Overall, 39 of 44 (88·6%) of our participants had a rhinovirus detected in respiratory samples during the 2-year follow-up. This was true whether or not participants were symptomatic.

Several recent studies have used similar molecular methods to evaluate respiratory viruses in sputum, nasal, or OP samples and found a far greater sensitivity for rhinovirus using RT-PCR compared with culture.[8,9,15–17] In a Danish study of children with CF followed for 1 year, rhinoviruses were detected at least once in more than 50% of patients.[9] It is not surprising that rhinoviruses are commonly detected in children with CF, as they are the most common viruses detected in similarly aged healthy pediatric populations [18] when improved PCR techniques are utilized.[14]

Rhinoviruses have been increasingly associated with substantial respiratory disease, causing hospitalization in young children and severe disease in immunocompromised hosts.[18–22] Rhinoviruses have also been associated with an increase in disease severity and lower respiratory symptoms in individuals with asthma compared with healthy controls.[23] Using clinical information collected during the study, we found that rhinorrhea and abnormal chest examination were associated with the rhinovirus detection; increased cough and sputum production were most predictive of rhinovirus among all symptoms reported by patients.

Exploratory analyses for our current CF cohort were inconclusive as to the association of rhinovirus with respiratory-related illness. This differs from the findings of Wat *et al.*[16]in the UK who detected rhinoviruses in 7·4% of samples obtained during routine clinic visits and in 15·9% of those obtained during an exacerbation ($P = 0·0027$), suggesting an association between rhinovirus and pulmonary exacerbations. These discrepant results may reflect our study design, with a smaller study population (44 versus 71 participants) and relatively less frequent sampling (quarterly versus every 2 months). In addition, the UK study included children between the ages 0 and 18, whereas our study excluded patients under 6 years of age. It might be expected that rhinoviruses were more commonly associated with pulmonary exacerbations when younger children were included. Studies in hospitalized children under five who do not have CF have demonstrated rates of rhinovirus-associated hospitalization of 4·8 per 1000 children, with much higher rates in those with a history of wheezing or asthma (25·3 hospitalizations pre-1000 children).[18]

The detection of rhinoviruses in both symptomatic and asymptomatic subjects raises the possibility of prolonged viral shedding in children with CF. Prolonged shedding in children with asthma, for example, has been previously reported in several studies.[23] However, because we only followed patients at routine quarterly clinical visits and because our techniques did not type the rhinoviruses, we cannot definitively document prolonged shedding versus multiple reinfections with single or different rhinovirus subtypes.

Some potential limitations of the current study include the relatively small sample size and the exclusion of young children who have the highest rates of viral prevalence. Sputum samples were used to assess viral shedding in the lower airway, as the use of an invasive procedure such as bronchoalveolar lavage could not be justified in our study. The question of contamination of sputum samples by respiratory viruses in the upper airway is another potential concern. However, the distribution of our concordant and discordant samples argues against this. Finally, the

frequency of sampling (that was chosen to reflect current clinical practice guidelines) was not sufficiently frequent to detect viral infections early in their course.

Our study provides further evidence that PCR-based viral detection allows for rapid and accurate diagnosis of viral infections in children with CF. Our results also suggest that samples from either upper airway or sputum perform well for virus detection. Rhinovirus was the most prevalent respiratory virus detected, as might be expected in children of this age. Recent studies demonstrating the important short-term and long-term impacts of rhinovirus in children with and without underlying lung disease emphasize the impact of this virus [18] and suggest a need to reexamine its impact in children and young adults with CF.

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