

## The Differential Clinical Impact of Human Coronavirus Species in Children With Cystic Fibrosis

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**We investigated the clinical impact of human coronaviruses (HCoV) OC43, 229E, HKU1 and NL63 in pediatric patients with cystic fibrosis (CF) during routine and exacerbation visits. A total of 408 nasopharyngeal aspirate samples were obtained from 103 patients over a 1-year period. Samples positive for HCoV were submitted for nucleotide sequencing to determine the species. Nineteen samples (4.65%) were positive for HCoV, of which 8 were positive for NL63, 6 for OC43, 4 for HKU1, and 1 for 229E. Identification of HCoV was not associated with an increased rate of respiratory exacerbations, but NL63-positive patients had higher exacerbation rates than patients who were positive for other HCoV species.**

Cystic fibrosis (CF) is an autosomal inherited disease characterized by recurrent and chronic respiratory infections [1]. The role of respiratory viruses in the progression of lung disease in CF is still controversial, although infection with respiratory syncytial virus (RSV) and influenza viruses was shown to carry a significant risk for respiratory exacerbations and hospital admissions [2].

While little is known of the impact of newly described respiratory viruses on lung disease in CF patients, there is

evidence that some of these viruses may be more aggressive than others. For example, we recently showed that infection with human rhinovirus C is associated with an increased risk of respiratory exacerbations [3].

Human coronaviruses (HCoVs) were initially described in the 1960s as the causative agents of upper respiratory tract infections (URTIs). Although reports of lower respiratory tract infections caused by HCoVs were identified thereafter [4], the coronaviruses were still mainly considered to be the causative agents of URTIs [5] until 2002, when a new coronavirus, later named SARS coronavirus, was identified during an outbreak of severe acute respiratory syndrome [6]. More recently, other coronaviruses were described, with HCoV-NL63 identified in the Netherlands in 2004 [7] and HCoV-HKU1 in Hong Kong in 2005 [8]. These novel HCoV species were hypothesized to cause lower respiratory tract infections at a higher frequency than the previously known HCoVs [9], although recent data suggest that HCoV-HKU1 does not cause a significantly higher proportion of LRTIs than that observed with HCoV-OC43 [10].

In this study, we aimed to investigate the clinical impact of all 4 circulating HCoVs (OC43, 229E, HKU1, and NL63) in pediatric patients with CF who attended an outpatient clinic during routine and exacerbation visits.

### METHODS

A total of 103 CF patients (49 females, 54 males; median age, 8.9 years [range, 3.8 months to 17.8 years]) were enrolled in this study from 6 September 2006 through 4 September 2007. Nasopharyngeal aspirates (NPAs) or nasal blow (NB) specimens for viral investigation, as well as sputum or oropharyngeal samples for microbiology cultures, were collected during scheduled or unscheduled visits on 408 occasions, with a median ( $\pm$  SD) of  $4 \pm 1.74$  visits (range, 1–9 visits) per patient.

The local ethics committee approved the study protocol, and all parents/guardians gave informed consent for their children to participate in the study.

Clinical and functional data were obtained at all visits. Exacerbation of respiratory disease was defined as the presence of  $\geq 2$  of the following symptoms and signs: fever, increase in the amount of secretion or cough intensity, change in the sputum's aspect, worsening of dyspnea, loss of appetite, a decrease of  $\geq 10\%$  in forced expiratory volume in 1 second (FEV1), and weight loss.

Total nucleic acids were extracted from nasopharyngeal samples with the QIAmp Viral RNA Mini Kit (Qiagen,

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Hamburg, Germany), according to the manufacturer's instructions. Reverse transcription was performed with a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA), using 20  $\mu$ L of previously extracted RNA. Identification of respiratory viruses was performed by individual polymerase chain reactions (PCRs) targeting RSV; influenza viruses A and B; human parainfluenza viruses 1, 2, and 3; HCoV; human metapneumovirus; adenovirus; human bocavirus; enteroviruses; and the  $\beta$ -actin gene, as previously described [3]. The analytical sensitivity of this assay for HCoV detection, determined by quantified plasmid standards, was 100 copies per reaction.

All coronavirus-positive samples were confirmed by means of 1-step reverse transcription–heminested PCR, using primers CoV2A-F (CTTATGGGTTGGGATTATCC) and CoV2A-R (TAATAACAGACAACGCCATCATC) for the first round and the inner primers CoV2A-Rnest a (CCATCATCACTCAGAATCATCA) and CoV2A-Rnest b (CCATCATCAGAAAGAATCA TCA), which generate a 404–base pair amplicon from the RNA-dependent RNA polymerase (RdRp) gene. Nucleotide sequencing was performed using dye terminator chemistry (Applied Biosystems). Nucleic acid alignment was conducted on the basis of the amino acid code by use of the BLOSUM algorithm. Phylogenetic classification was performed using the neighbor-joining method, with the percentage distance substitution model and bootstrap values calculated from 1000 replicates with MEGA 5.0 software ([www.megasoftware.net](http://www.megasoftware.net)). The nucleotide sequences of the partial RdRp gene of the HCoV identified in this study were submitted to GenBank (accession numbers: JN251784 - JN251802).

The results were expressed as medians or percentages, unless stated otherwise. Categorical variables were analyzed by  $\chi^2$  or Fisher exact tests. The age differences between patients with and patients without HCoV or within HCoV species were compared by nonparametric Kruskal–Wallis tests. The proportion of cases with acute respiratory exacerbation among patients infected with different HCoV species was compared by  $\chi^2$  or Fisher exact tests.

Logistic regression was used to determine whether infection with HCoV in general or with one particular HCoV species was independently associated with respiratory exacerbation or hospital admission. To account for correlations among samples within the same subject, binomial generalized linear models were used. All analyses were performed using SPSS for Windows (version 18; SPSS, Chicago, IL), and the level of statistical significance was set at .05.

## RESULTS

At least 1 respiratory virus was identified in 203 of 408 samples (49.7%), with rhinovirus being the main identified agent (in 139 samples [34.1%]). HCoVs were identified in 19 samples (4.65%) from 17 patients (11 males and 6 females).

As shown in Figure 1, 8 of the viruses were NL63, 6 were OC43, 4 were HKU1, and 1 was 229E. Two patients had 2 separate identifications of HCoV in samples collected 2 and 6 months apart, respectively, and different species were identified on each occasion. Single HCoV infections were found on 13 occasions, while coinfections were observed on 6 occasions, four with the identification of 2 viruses (rhinovirus or enterovirus) and two with 3 viruses identified (RSV + rhinovirus and influenza A virus + enterovirus).

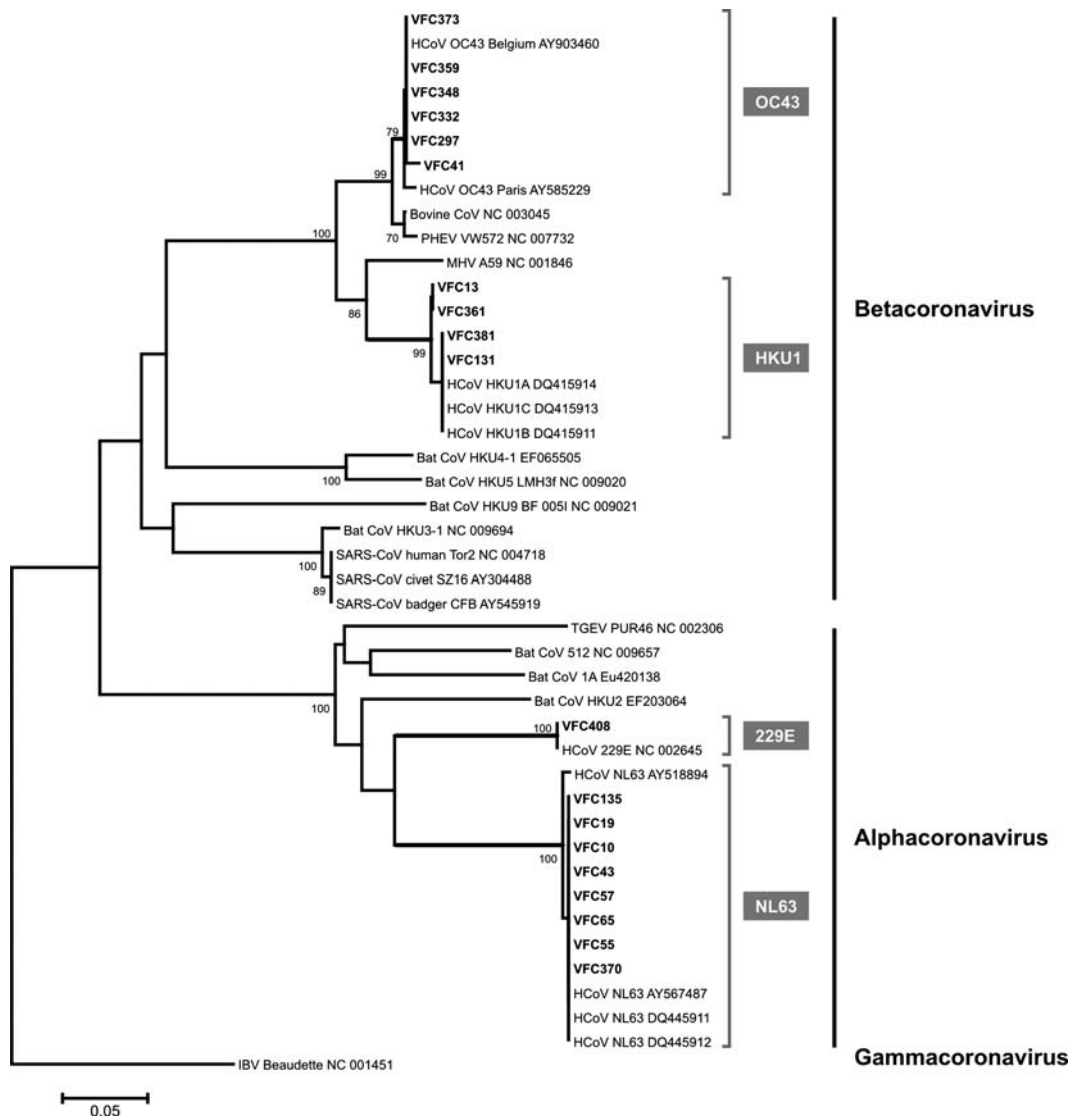
Overall, 142 episodes (34.8%) of respiratory exacerbations were identified, and hospital admissions occurred in 31 (7.6%) of them. Significant associations were found for an increased risk of respiratory exacerbation when rhinovirus C was identified and an increased risk of hospital admission with influenza virus infections (data not shown). No significant difference was found among patients with HCoV infection, with exacerbations identified on 6 of 19 occasions (31.5%) and hospital admissions on 2 of 19 occasions (10.5%).

The HCoV species that were found are displayed in Table 1, as are patient characteristics, including the frequency of symptoms and the clinical status at the time of the visit. The age distribution was similar among patients infected with different HCoV species, although a significant difference was found among the ages of patients without viral infections and those infected with HCoV or other viruses (Table 1). Although no significant differences were seen concerning reported symptoms and clinical findings among patients infected with different HCoV species, a significantly greater respiratory exacerbation rate was found among patients infected with HCoV-NL63 as compared to those infected with other HCoV species ( $P = .04$ ). In the logistic regression model, however, neither the identification of any HCoV nor HCoV-NL63 specifically could be demonstrated as being significantly associated with a respiratory exacerbation.

## DISCUSSION

This is the first description of the identification of these new HCoV species among patients with CF. While no significant clinical impact could be attributed to infection with these species as a whole, the identification of HCoV-NL63 was associated with a significantly higher rate of respiratory exacerbation as compared to other HCoV species.

Although HCoVs were previously identified as causative agents of lower respiratory tract infections, the medical community commonly knew them as “common cold” viruses. The identification of new species of HCoV [7, 8] and the emergence of SARS HCoV [6] highlighted the potential role of these viruses as causative agents of severe lower respiratory tract infections. Following initial descriptions of these new “non-SARS” HCoV, several other studies describing the prevalence and clinical impact were published, although results



**Figure 1.** Representative neighbor-joining phylogenetic tree of a partial RNA-dependent RNA polymerase region of coronavirus (CoV), generated with a p-Distance model on the basis of a 134-amino acid sequence by use of MEGA 5.0 software (available at: <http://www.megasoftware.net>). Reference CoV species data were obtained from the GenBank database. Viruses identified in this study are indicated in boldface and labeled VFC. Bootstrap values >70% in the key branches are depicted. Scale bar indicates amino acid substitutions per site. The strain designations, GenBank accession numbers, and corresponding hosts are indicated. The respective genera are given on the right. The highly conserved amino acid sequence fragment did not permit differentiation of the 2 NL63 CoV clusters.

were conflicting. While the prevalence values of these new HCoV-NL63 and HCoV-HKU1 oscillate from 0.4% to 9% [5], reports of severe cases of lower respiratory infections were described in Australia for the HCoV-NL63 [11] and in Germany for the HCoV-HKU1 [12].

However, most of the studies on the clinical impact of different HCoV species were only performed among children who were hospitalized for acute respiratory tract infections, with small sample sizes and short periods of sample collection [10]. More recent studies have included much larger samples. Gaunt et al. [9], who studied 11 600 respiratory samples

obtained between 2006 and 2009 in Edinburgh, United Kingdom, found at least 1 coronavirus in 2.3% of the samples, with marked predominance in winter months. Although they found a significant number of coinfections (mainly with RSV), single infections with HCoV-OC43, HCoV-NL63, and HCoV-HKU1 were specifically associated with lower respiratory tract disease. They also observed that HCoV-229E, which was identified in a smaller proportion of samples, was predominant among immunosuppressed patients. Talbot et al. [10] used molecular tools to determine the incidence and clinical features of upper and lower respiratory tract infections that are

**Table 1. Clinical Characteristics, According to the Virological Results, for 408 Clinical Visits Involving 103 Children With Cystic Fibrosis**

	HCoV-NL63 (n = 8)	HCoV-OC43 (n = 6)	HCoV-HKU1 (n = 4)	Any HCoV <sup>a</sup> (n = 19)	Other Viruses (n = 184)	No Virus (n = 205)
Median age (mo)	67.96	66.95	73.46	77.46	89.40	119.00 <sup>b</sup>
Male sex	6 (75)	5 (83.3)	2 (50)	13 (68.4)	106 (57.6)	101 (49.3)
Lack of appetite	2 (25)	2 (33.3)	0	4 (21)	28 (15.2)	22 (10.7)
Weight loss	1 (12.5)	1 (16.6)	0	2 (10.5)	23 (12.5)	28 (13.6)
Increase of amount of sputum	5 (62.5)	1 (16.6)	0	6 (31.5)	73 (39.6)	60 (29.2)
Change of sputum's aspect	4 (50)	0	0	4 (21)	43 (23.3)	29 (14.1)
Worsening of dyspnea	2 (25)	1 (16.6)	0	3 (15.8)	41 (22.3)	38 (18.5)
FEV1 decrease ( $\geq 10\%$ ) <sup>c</sup>	0	0	0	1 (5.3)	11 (5.9)	17 (8.3)
Fever	1 (12.5)	0	0	1 (5.3)	23 (12.5)	25 (12.2)
Coryza	5 (62.5)	2 (33.3)	1 (25)	9 (47.4)	59 (32.1)	41 (20)
Sore throat	2 (25)	1 (16.7)	0	3 (15.8)	18 (9.8)	15 (7.3)
Diarrhea	0	0	0	1 (5.3)	7 (3.8)	11 (5.4)
Wheezing	0	0	0	0	16 (8.7)	12 (5.9)
Stridor	0	0	0	0	1 (0.5)	1 (0.5)
Exacerbation	5 (62.5)	1 (16.7)	0	6 (31.6)	72 (39.1)	64 (31.2)
Admission	1 (12.5)	1 (16.7)	0	2 (10.5)	16 (8.7)	13 (6.3)

Data are no. (%) of visits, unless otherwise indicated.

Abbreviations: FEV1, forced expiratory volume in 1 second; HCoV, human coronavirus.

<sup>a</sup> Including 1 case of HCoV-229E infection.

<sup>b</sup> Significantly different from values for the HCoV-positive groups ( $P = .03$ , by the Kruskal–Wallis) and from the value for the “Other Viruses” group ( $P = .001$ , by the Mann–Whitney  $U$  test).

<sup>c</sup> Results were available for only 227 of 408 clinical visits.

associated with HCoV-NL63, HCoV-OC43, and HCoV-229E during a 20-year period and showed that HCoV-OC43 and HCoV-NL63 were associated with a significant burden of lower respiratory tract infections in previously healthy outpatient children who manifested with clinical syndromes, such as pneumonia and bronchiolitis, that were similar to those caused by other respiratory viruses [10].

Patients with CF represent a specific population that is susceptible to more severe clinical manifestations of respiratory infections overall, because of limitations in mucociliary transport and anatomic distortions of the bronchial tree that facilitate the retention and infection of respiratory secretions. The impact of respiratory viruses in CF lung disease has been recently reassessed using molecular methods by some researchers, with conflicting results: while Olesen et al [13] reported a lack of significant clinical impact of viral infections in lung function or respiratory symptoms, Wat et al [2] described an association of viral infections with respiratory exacerbations in CF children, particularly when influenza A virus, influenza B virus, and rhinovirus infections were identified.

One possible explanation for the differential clinical impact of respiratory viruses for CF patients is the baseline lung function, and it is possible that patients with worse lung disease would be at more risk to present an exacerbation; we cannot

rule out this possibility in the present study, because of the small number of patients infected with HCoV. Another hypothesis is related to the mechanisms of disease of HCoV. Both SARS coronavirus and HCoV-NL63 use the angiotensin-converting enzyme 2 (ACE2) as the main pathway for attachment and entry. Following infection, a substantial reduction of the ACE2 expression in the cell surfaces occurs [14], but the rate and the intensity of this downregulation seems to be much lower in HCoV-NL63 infections as compared to SARS coronavirus infections. ACE2 downregulation was previously shown to be associated with an increase of the inflammatory process in the respiratory tract and may be one of the determinants of severity in acute respiratory distress syndrome [14]. Moreover, genetic polymorphisms associated with an increase of ACE expression (which counterbalance ACE2 action as a proinflammatory mediator) were shown to be associated with worse lung disease in CF patients [15].

We have previously shown that human rhinovirus C infections were associated with an increased risk of respiratory exacerbations in CF children [3], and although we could not identify a significant association of HCoV infections with respiratory exacerbations in the present analysis, such exacerbations were observed in a significant proportion (62.5%) of HCoV-NL63–infected children. It should be mentioned that

the mean age of our patients was approximately 9 years, while in the study by Talbot et al. [10], the greatest incidence of LRI associated with these viruses occurred between 6 and 23 months of age. Therefore, a study of infants with CF could produce very different results.

In conclusion, we found all circulating species of HCoV in children with CF who were enrolled in this study. Symptoms were mainly related to the upper airway, and we did not find an association between HCoV infections in general or infections with a given HCoV species and an increased risk of respiratory exacerbation or hospital admission, although a significantly higher rate of respiratory exacerbation was observed among HCoV-NL63-infected children as compared to children infected by other coronaviruses.

## Notes

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