Infections by Human Coronavirus-NL in Hospitalized Children

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Background: A new human coronavirus (HCoV), HCoV-NL, was recently reported for Dutch patients with acute respiratory tract infections (ARTI). Little information is available on the incidence, clinical manifestations and epidemiologic features of HCoV-NL infections.

Methods: We performed a prospective study of symptomatic (case subjects with ARTI) and asymptomatic (control subjects undergoing elective surgery) children, ≤3 years of age, hospitalized in Canada during 2 winter seasons (2001–2003), to look at the prevalence of respiratory viruses. Reverse transcription-PCR assays were used to retrospectively detect HCoV-NL and to correlate its presence with clinical symptoms.

Results: HCoV-NL was detected in nasopharyngeal aspirates from 3.0% of young children (12 of 396 children) hospitalized for treatment of ARTI (case subjects), compared with 1.7% of asymptomatic control subjects (3 of 177 children) ($P = 0.6$). Nine (75.0%) of the symptomatic children had mixed viral infections. The mean age and mean duration of hospitalization of case subjects were 10.1 months and 4.9 days, respectively. Final diagnoses consisted of bronchiolitis or bronchitis (9 of 12 cases), pneumonitis (1 of 12 cases) and upper respiratory tract infections (2 of 12 cases), although 2 of 3 subjects with single HCoV-NL infections had upper respiratory tract infections only. Sequence analysis of the 1a and spike genes revealed that multiple HCoV-NL strains circulated in the same geographical area in each of the 2 winter seasons. Variability was more pronounced for the spike gene, with 2 clusters of strains.

Conclusions: HCoV-NL was not a major respiratory pathogen in Canada during our study, as shown by its low detection rate in hospitalized children with ARTI, coupled with the high frequency of additional pathogens and its occasional detection in healthy children.

Key Words: coronavirus-NL, acute respiratory tract infections, pediatric infections, polymerase chain reaction, phylogenetic analysis

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The coronaviruses (CoVs) (order Nidovirales, family Coronaviridae, genus *Coronavirus*) are large, positive-stranded, RNA viruses responsible for pulmonary, gastrointestinal and neurologic diseases in domestic and wild animals.[1] The first 2 CoVs identified in human subjects [human CoV (HCoV)-229E and HCoV-OC43] have been traditionally associated with common colds in adults[2] and, more recently, with clusters of acute infections of the upper and lower respiratory tracts in all age groups.[3] In 2003, the large outbreak of cases of severe acute respiratory syndrome (SARS) led to the discovery of a new CoV, the SARS-CoV.[4,5] Although first identified in human subjects, it is likely that the SARS-CoV agent has an animal reservoir.[6,7] In 2004, a fourth HCoV (HCoV-NL) was reported simultaneously by 2 groups in the Netherlands.[8,9] Subsequently this virus and a closely related virus designated New Haven HCoV (HCoV-NH) were reported in North America.[10–12] The nucleotide sequence of the complete genome indicates that HCoV-NL is a member of CoV group 1, being most closely related to the porcine epidemic diarrhea virus and HCoV-229E.[8,9,13]

Little clinical information is available about HCoV-NL infections. In one study by van der Hoek et al,[8] the virus was initially identified in a nasopharyngeal aspirate (NPA) from a 7-month-old child who had bronchiolitis with conjunctivitis. A retrospective study from the same group identified 7 additional cases, involving 4 young children (<1 year) and 3 adults (2 of whom were immunocompromised), during the winter of 2003 in Amsterdam.[8] In a study by Fouchier et al,[9] the virus was initially isolated from a nose swab collected from a 8-month-old boy with pneumonia. HCoV-NL was also detected in 4 (2.9%) of 139 respiratory tract specimens that tested negative for known viruses between November 2000 and January 2002 in Rotterdam. In a Canadian study, HCoV-NL was detected in 3.6% of respiratory samples (19 of 525 samples) sent to a reference laboratory with very limited clinical information available on infected subjects (mostly outpatients).[10] In this study, we sought to evaluate the incidence, epidemiologic features and clinical manifestations of

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HCoV-NL in a well-defined population that consisted of hospitalized children with acute respiratory tract infections (ARTI) (case subjects) and others admitted for elective surgery (control subjects) during 2 consecutive winter/spring seasons in Quebec, Canada.

METHODS

Study Design. The design of this study, aimed at elucidating the cause of ARTI in children, has been reported elsewhere.[14] Briefly participants consisted of children ≤3 years of age who were hospitalized between December 15, 2001, and April 20, 2002 (year 1), and between January 15, 2003, and May 29, 2003 (year 2), at a single hospital in Quebec City, Canada. Case subjects were children admitted because of an ARTI episode. Eligible control subjects were children who were hospitalized for any elective surgery during the same periods (without more specific matching) and who had no respiratory symptoms or fever at the time of sample collection. At admission, a research nurse obtained signed consent from parents before collecting a NPA specimen (typically ~2 mL per suction) and completing a specific questionnaire for each child. An aliquot of the NPA samples was immediately processed for viral cultures and the rest was frozen at −80°C for future reverse transcription (RT)-PCR tests. The nurse also reviewed all clinical and laboratory data in the children's clinical charts at the end of hospitalization. The study was approved by the Centre Hospitalier Universitaire de Québec research ethics board.

Virologic Testing. All NPA specimens from case and control subjects were first prospectively tested with real-time RT-PCR assays for influenza A and B, human respiratory syncytial virus (hRSV) and human metapneumovirus, as previously described.[14] In addition, all NPA specimens from case subjects were prospectively tested for the presence of hRSV antigens with the RSV TestPack (Abbott Laboratories, Abbott Park, IL). Viral cultures (notably for orthomyxoviruses, paramyxoviruses, enteroviruses and adenoviruses) were performed at the request of the treating physician in 326 (82.3%) of 396 cases, as reported.[14]

Extracted RNA or complementary DNA aliquots (kept at −80°C) were retrospectively tested for the new HCoV-NL virus ~1–2.5 years after specimen collection. Samples were initially tested for HCoV-NL with real-time PCR assays using the primers and TaqMan probe (set 3) reported by Fouchier et al,[9] in a LightCycler instrument (Roche Diagnostics, Laval, Quebec, Canada). This set of primers amplifies a 99-nucleotide fragment in the viral nucleocapsid gene. The real-time PCR assay for the nucleocapsid gene detected 100 copies of a transcribed viral plasmid, with no positive signals obtained after testing of hRSV ($n = 4$), influenza A ($n = 3$), HCoV-229E ($n = 1$) and HCoV-SARS ($n = 1$) RNA samples. All PCR-positive samples were subsequently tested with another PCR protocol for gene 1a of HCoV-NL, as reported.[8] Finally a new real-time PCR for the HCoV spike gene was designed with the following primers: 5′-ATGAAACTTTTCTTGATTTTGC-3′ (forward) and 5′-GAAGATTTAAGACCAGGTACAGG-3′ (reverse). Nucleotide sequences of the 1a and spike genes were used to construct phylogenetic trees based on the neighbor-joining algorithm and Kimura-2 parameters.

RESULTS

PCR Positivity Rates for HCoV-NL. Overall, >90% of eligible patients (case and control subjects) were recruited in the study. With the real-time PCR assay for the nucleocapsid gene, 3.0% of samples (12 of 396 samples) from children with ARTI (case subjects) tested positive for HCoV-NL during the 2 winter/spring seasons, compared with 1.7% of samples (3 of 177 samples) from children admitted for elective surgery (control subjects) ($P = 0.6$, Fisher's exact test). HCoV-NL was detected more often in the first year of the study (2001–2002), when 4.7% of samples (9 of 193 samples) from case subjects were positive, compared with 0 of 51 samples from control subjects ($P = 0.2$, Fisher's exact test). The PCR positivity rate was lower and similar between case subjects (3 of 203 samples, 1.5%) and control subjects (3 of 126 samples, 2.4%) during the second year of the study (2002–2003). No other viruses were detected with PCR for the 3 HCoV-NL-positive control subjects, but 9 of 12 (75.0%) HCoV-NL-positive case subjects had mixed viral infections, as determined with a combination of PCR, viral culture and antigen detection methods. These mixed infections also included hRSV ($n = 4$), hRSV plus adenovirus ($n = 1$), hRSV plus influenza A ($n = 1$), influenza A ($n = 2$) and adenovirus ($n = 1$). Among the 15 samples determined to be HCoV-NL positive with real-time PCR for the nucleocapsid gene, 14 (93.3%) were also positive by conventional PCR for the 1a gene, whereas 11 (73.3%) were positive for the spike gene. Cycle threshold values, which are proportional to the amounts of viral RNA, did not differ significantly between PCR-positive case subjects and control subjects (data not shown).

Clinical Findings for HCoV-NL-Positive Patients. The distribution of HCoV-NL-positive case subjects and control subjects differed during the 2 years of the study. In 2001–2002, all HCoV-NL-positive samples were recovered between January 7 and March 21, whereas all positive samples from 2002–2003 were found between March 22 and April 22. Clinical findings for HCoV-NL-positive case subjects and control subjects are summarized in Table 1. The mean age of the 15 HCoV-NL-positive patients was 12.9 months; more specifically it was 10.1 months for the 12 case subjects and 24.3 months for 3 control subjects ($P = 0.04$, Kruskal-Wallis test) and 11.2 months for the 9 case subjects with mixed infections, compared with 6.8 months for the 3 case subjects with single HCoV-NL infections ($P = 0.4$). Five (33.3%) of 15 HCoV-NL-positive patients were male. The rest of the clinical findings were analyzed only for the 12 positive children hospitalized for treatment of ARTI (case subjects). An underlying disease, consisting of refractory cardiac arrhythmia, was present in 1 case. Among the clinical symptoms, all case subjects had nasal congestion and cough, 7 had fever, 5 had wheezing, 4 had otalgia or diarrhea and 3 had sore throat. The mean duration of hospitalization of case subjects was 4.9 days (median, 5 days; range, 2–10 days). The mean hospitalization stay was not statistically different for case subjects with mixed viral infections (4.8 days) and those with single HCoV-NL infections (5.3 days) ($P = 0.6$,

TABLE 1. Clinical Findings for HCoV-NL-Positive Children in Canada

Strain No.	Year of Isolation	NPA Date	Age (mo)	Gender	Underlying Disease	Final Diagnosis	Hospital Stay (d)	Other Viruses
14	2002	January 7	9	F	Yes*	URTI	7	—
24	2002	January 9	4	F	No	Bronchiolitis, otitis	5	hRSV, FluA
69	2002	January 22	32	M	No	Pneumonitis, otitis	5	FluA
110	2002	February 9	13	M	No	Bronchitis, otitis	3	Adeno
127	2002	February 15	1	M	No	Bronchiolitis, otitis	6	hRSV
131	2002	February 17	20	F	No	Bronchitis, Kawasaki disease	5	FluA
156	2002	February 27	6	F	No	Bronchiolitis, otitis	2	hRSV
172	2002	March 11	0.5	M	No	URTI	4	—
191	2002	March 21	1	F	No	Bronchiolitis	5	hRSV
157	2003	March 22	13	F	No	Bronchospasm	10	hRSV, adeno
169	2003	March 31	11	F	No	Bronchiolitis, otitis	5	—
175	2003	April 2	11	F	No	Bronchiolitis, otitis	2	hRSV
C35†	2003	March 3	20	F	No	Myringotomy	—	—
C54†	2003	April 1	24	M	No	Myringotomy	—	—
C79†	2003	April 22	29	F	No	Tonsillectomy	—	—

*Refractory cardiac arrhythmia.

†Denotes controls, ie, children hospitalized for an elective surgery without fever and respiratory symptoms.

NPA indicates nasopharyngeal aspirate; Flu A, influenza A virus; adeno, adenovirus.

Kruskal-Wallis test). Similarly it was not different for those with mixed hRSV and HCoV-NL infections (3.8 days) and single hRSV infections (4.9 days) ($P = 0.5$). One patient with a mixed HCoV-NL, hRSV and adenovirus infection required a stay in the intensive care unit, and none of the case subjects died. Final diagnoses for the 12 case subjects are reported in Table 1. Although 9 of 12 case subjects had a diagnosis of bronchiolitis or bronchospasm or bronchitis, upper respiratory tract infection (URTI) only was found for 2 of 3 case subjects with single HCoV-NL infections. Kawasaki disease was diagnosed for 1 patient (patient 131) coinfected with influenza A and HCoV-NL and also for 1 HCoV-NL-negative child enrolled in the study.

Phylogenetic Analysis of HCoV-NL Strains. Sequences (425 bp) of the 1a gene of 14 HCoV-NL strains from the present study, along with a strain recovered from an adult in 2004 (no. 95665) and other strains from the Netherlands[8] and Canada[10] reported in GenBank, were aligned with the prototype strain nl63.[8] The sequenced 1a gene region from our strains was well conserved, with nucleotide and amino acid identities of 98.2–100% and 98.0–100%, respectively. Different strains of HCoV-NL cocirculated in Quebec (Canada) in 2002 and 2003. However, similar 1a sequences were found in Canada and in the Netherlands during the same period. Sequences (602 bp) of the spike gene of 11 strains were also aligned with the prototype strain nl63. The nucleotide and amino acid identities for the spike gene were 90.3–100% and 87.5–100%, respectively. Two clusters of viral strains were found with phylogenetic analysis (data not shown).

DISCUSSION

We found the presence of the newly reported HCoV-NL in 3% of young children hospitalized for treatment of ARTI during the winter/spring months over 2 consecutive years in Canada but also in 1.7% of children who were undergoing elective surgery during the same period and who had no fever or respiratory symptoms at the time of sample collection. As a comparison, we found much higher incidence rates for hRSV (50.2%), influenza A or B viruses (12.7%) and human metapneumovirus (5.5%) in the same cohort of patients with similar real-time PCR methods.[14,15] We detected another viral pathogen for 60% of all HCoV-NL-positive children and 75% of HCoV-NL-positive children with ARTI. The main diagnoses associated with this new viral infection were bronchiolitis/bronchitis for 75% of case subjects, although sick children with single HCoV-NL infections had more URTIs than bronchiolitis.

The incidence and role of HCoV-NL in causing ARTI have not been well studied. In 2 small retrospective studies from the Netherlands, HCoV-NL was detected with RT-PCR in 7% of respiratory specimens (5 of 70 specimens) from hospitalized and outpatient subjects in January 2003[8] and in 2.9% of respiratory specimens (4 of 139 specimens) from individuals with ARTI between November 2000 and January 2002.[9] In larger laboratory-based studies from North America, the incidence of HCoV-NL/HCoV-NH infections ranged from 3.6% in individuals of all ages[10] to 8.8% in children <5 years of age.[11] More than one-half of the HCoV-positive children in the latter study also had important underlying diseases, which might have predisposed them to more severe respiratory diseases. However, the previous studies had some limitations, including their retrospective design, the exclusion of samples that tested positive for other viruses, the inclusion of subjects from different age groups and/or different clinical settings (hospitalized patients and outpatients) and the incomplete description of the clinical findings associated with HCoV-NL infections. Recently the presence of HCoV-NH was also associated with Kawasaki disease in a small, retrospective, case-control study, although viral detection in inflamed tissues was not sought.[12]

Our data derived from a prospectively designed, case-control study in a well-defined population (hospitalized children ≤ 3 years of age) differ from those of previous reports in some aspects. Although the incidence of HCoV-NL infections in our population was similar to that reported by Fouchier et al[9] (3% versus 2.9%), we found a high rate of coinfecting viral pathogens, most notably hRSV in 50% of cases. In addition, an underlying disease was present in only

1 of 12 HCoV-NL-positive cases. This new HCoV was detected in 3 (1.7%) of 177 control children, in contrast to the lower rates of detection of hRSV, influenza and human metapneumovirus for the same asymptomatic children.[14] We cannot speculate whether viral detection in these control subjects represents true asymptomatic infection, as reported for volunteers inoculated with HCoV-229E,[16] or prolonged viral excretion after ARTI, because our subjects were not questioned about prior respiratory symptoms. Also, we cannot exclude a more important role for HCoV-NL for other subjects, particularly those with severe underlying diseases. Although 1 of our HCoV-NL case subjects had a diagnosis of Kawasaki disease,[12] our study was not designed to look at such association. The fact that single HCoV-NL symptomatic infections in our study occurred at an earlier age than did single HCoV-NL asymptomatic infections (mean of 6.8 months versus 24.3 months) may indicate that primary infections are relatively more severe when they occur earlier in life. Two of the 3 symptomatic children with pure HCoV-NL infections presented with URTIs, in agreement with studies of HCoV-OC43 and HCoV-229E infections.[1,3] In addition, >50% of all HCoV-NL case subjects in our study had concomitant otitis media, which was previously reported for other HCoVs.[3]

Our 2-year study sheds some light on HCoV-NL epidemiologic features. Infections occurred during both years of the study, with all cases from year 1 occurring during the winter season, in contrast to the spring cases of year 2. Infections with other HCoVs have been reported to follow a cyclical pattern, with outbreaks occurring every 2–4 years.[17–19] Although we did not look for the presence of HCoV-NL during the summer and fall months, preliminary data from the Netherlands indicate that circulation of this virus is not frequent during this period.[8,9] On the basis of sequence analysis of the 1a and spike genes, which code for a large nonstructural polyprotein and the major envelope protein, respectively, we found that more than 1 HCoV-NL strain can circulate in the same geographic area during the same period of time. Of interest, some Canadian 1a sequences were identical with those from the Netherlands, reinforcing the conservation of this gene. We found more variability in the spike gene (with 2 clusters of strains), which may suggest that this envelope protein responsible for cell binding is under immune pressure.

In conclusion, we found that the newly described HCoV-NL was not a major respiratory viral pathogen during our study, as shown by its low detection rate in hospitalized children, the high frequency of concomitant viral pathogens and its occasional detection in asymptomatic children. Considering only single infections, HCoV-NL was not found more frequently in children with ARTI, compared with asymptomatic children admitted for surgery. Prospective and population-based studies with appropriate control groups are still needed to establish causal relationship between this virus and various respiratory and nonrespiratory clinical syndromes.

REFERENCES

1. Holmes KV. Coronavirus. In: Knipe DM, Howley PM, eds. *Fields Virology*. 4th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2001:1187–1203.
2. Hendley JO, Fishburne HB, Gwaltney JMJ. Coronavirus infections in working adults: eight-year study with 229E and OC43. *Am Rev Respir Dis*. 1972;105:805–811.
3. Vabret A, Mourez T, Gouarin S, Petitjean J, Freymuth F. An outbreak of coronavirus OC43 respiratory infection in Normandy, France. *Clin Infect Dis*. 2003;36:985–989.
4. Drosten C, Gunther S, Preiser W, et al. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med*. 2003;348:1967–1976.
5. Poutanen SM, Low DE, Henry B, et al. Identification of severe acute respiratory syndrome in Canada. *N Engl J Med*. 2003;348:1995–2005.
6. Guan Y, Zheng BJ, He YQ, et al. Isolation and characterization of viruses related to the SARS coronavirus from animals in southern China. *Science*. 2003;302:276–278.
7. Martina BE, Haagmans BL, Kuiken T, et al. Virology: SARS virus infection of cats and ferrets. *Nature*. 2003;425:915.
8. van der Hoek L, Pyrc K, Jebbink MF, et al. Identification of a new human coronavirus. *Nat Med*. 2004;10:368–373.
9. Fouchier RA, Hartwig NG, Bestebroer TM, et al. A previously undescribed coronavirus associated with respiratory disease in humans. *Proc Natl Acad Sci USA*. 2004;101:6212–6216.
10. Bastien N, Anderson K, Hart L, et al. Human coronavirus NL63 infection in Canada. *J Infect Dis*. 2005;191:503–506.
11. Esper F, Weibel C, Ferguson D, Landry ML, Kahn JS. Evidence of a novel human coronavirus that is associated with respiratory tract disease in infants and young children. *J Infect Dis*. 2005;191:492–498.
12. Esper F, Shapiro ED, Weibel C, Ferguson D, Landry ML, Kahn JS. Association between a novel human coronavirus and Kawasaki disease. *J Infect Dis*. 2005;191:499–502.
13. Pyrc K, Jebbink MF, Berkhout B, van der Hoek L. Genome structure and transcriptional regulation of human coronavirus NL63. *Virol J*. 2004;1:7–17.
14. Boivin G, De Serres G, Cote S, et al. Human metapneumovirus infections in hospitalized children. *Emerg Infect Dis*. 2003;9:634–640.
15. Boivin G, Cote S, De Serres G, Gilca R, Bergeron MG, Dery P. Role of human metapneumovirus and other common respiratory viruses in children's hospitalizations for acute respiratory tract infection: a two-year study using multiplex PCR. In: *Program and Abstracts of the 43rd Interscience Conference on Antimicrobial and Chemotherapy (Chicago)*. Washington, DC: American Society for Microbiology; 2003:491. Abstract V-478.
16. Bradburne AF, Bynoe ML, Tyrrell DA. Effects of a "new" human respiratory virus in volunteers. *Br Med J*. 1967;3:767–769.
17. Monto AS, Lim SK. The Tecumseh study of respiratory illness, VI: frequency of and relationship between outbreaks of coronavirus infection. *J Infect Dis*. 1974;129:271–276.
18. Vallet S, Gagneur A, Talbot PJ, Legrand MC, Sizun J, Picard B. Detection of human coronavirus 229E in nasal specimens in large scale studies using an RT-PCR hybridization assay. *Mol Cell Probes*. 2004;18:75–80.
19. McIntosh K, Kapikian AZ, Turner HC, Hartley JW, Parrott RH, Chanock RM. Seroepidemiologic studies of coronavirus infection in adults and children. *Am J Epidemiol*. 1970;91:585–592.