Human Coronavirus NL63 Infection in Canada

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The isolation of human coronavirus NL63 (HCoV-NL63) in The Netherlands raised questions about its contribution to respiratory illness. In this study, a total of 525 respiratory specimens, collected in Canada primarily during the winter months of 2001–2002, were tested for HCoV-NL63; 19 tested positive for HCoV-NL63, demonstrating virus activity during January–March 2002. Patients with HCoV-NL63 were 1 month–100 years old (median age, 37 years). The main clinical presentations were fever (15/19), sore throat (5/19), and cough (9/19), and 4 patients were hospitalized. These results provide evidence for the worldwide distribution of HCoV-NL63.

Viral respiratory diseases are a major health problem and represent the leading cause of death due to infectious disease in Canada [1]. They affect people of all ages and exert a great economic impact on the health-care system. The viruses most frequently associated with respiratory-tract infections include rhinoviruses, coronaviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses (RSVs), adenoviruses, and the recently identified human metapneumovirus (hMPV). However, the etiological agents for a large number of respiratory infections remain unknown. A new human coronavirus, NL63 (HCoV-NL63), has been discovered in The Netherlands [2]. The virus was identified in clinical specimens from both infants and adults suffering from respiratory-tract illnesses [2]. Other HCoVs have been associated with respiratory illnesses. In certain populations, the most common HCoVs, HCoV-229E and HCoV-OC43, are responsible for up to 30% of the common-cold syndrome [3]. However, several studies have found that these viruses are associated with more-severe respiratory infections [4–7]. A novel coronavirus, HCoV-SARS, has been associated with severe atypical pneumonia and caused 774 deaths worldwide during November 2002–July 2003 [8]. Coronaviruses are divided into 3 serotypes, on the basis of both their host range and their genome sequence. Group 1 and 2 viruses are mammalian coronaviruses and include HCoV-229 and HCoV-OC43, respectively, whereas group 3 consists of avian coronaviruses. HCoV-SARS does not closely resemble viruses in any of the 3 known groups of coronaviruses. Analyses of the complete genome sequence of HCoV-NL63 have revealed that the virus is a new group 1 coronavirus that is closely related to HCoV-229 [2]. The relative importance of HCoV-NL63 in viral respiratory-tract illnesses is still not known. In the present study, we retrospectively looked for the presence of HCoV-NL63 in Canadian patients suffering from acute respiratory-tract infection (ARI) during the winter months of 2001–2002, to assess the impact that HCoV-NL63 infections have on ARI and to describe the presenting signs and symptoms of the ARI caused by this virus.

Materials and methods. Primers used for amplification and sequencing were based on the published HCoV-NL63 genome sequence [2]. Two nested sets of primers, described by van der Hoek et al. [2], were used in this study. The primer set based on the 1b gene—primers repSZ-1f-(15973) (5′-GTGATGCATATGCTAATTTG-3), repSZ-3r-(16210) (5′-CTCTTGCAGGTATAATCCTA-3′), repSZ-2f-(16012) (5′-TTGGTAAACAAAAGATAACT-3′), and repSZ-4r-(16181) (5′-TCAATGCTATAAACAGTCAT-3′)—were used for diagnosis; the primer set based on the 1a gene—primers SS5852-5Pf-(5777) (5′-CTTTTGATAACGGTCACTATG-3′), P4G1M-5-3Pr-(6616) (5′-CTCATTACATAAAACATCAAACGG-3′), P3E2-5Pf-(5788) (5′-GGTCACTATGTAGTTTATGATG-3′), and SS6375-3Pr-(6313) (5′-GGTCACTATGTAGTTTATGATG-3′)—were used for confirmatory purposes and sequence analysis. The primers used for amplification of the RNase P housekeeping gene were based on the published sequences (GenBank accession number NM_006413) 5′-AGATTTGGACCTGCGAGCG-3′ (forward primer) and 5′-GAGCGGCTGTCTCCACAAGT-3′ (reverse primer).

Viral RNA was extracted from 100 μL of either original samples or tissue-culture fluid, by use of the RNeasy Mini Kit (QIAGEN). Viral RNA was amplified by use of a 1-step reverse transcription–polymerase chain reaction (RT-PCR) kit (QIAGEN), according to the manufacturer's recommendations. In brief, 5 μL of RNA was added to the RT-PCR mixture, which

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Table 1. Data from medical files of patients infected with human coronavirus NL63.

Specimen	Date collected	Province	Specimen source	Characteristics of patients			
				Sex	Status	Age	Symptom(s)
33	11 January 2002	Saskatchewan	NPS	M	Outpatient	11 months	Fever, rash, lymphadenitis, sore throat
39	12 January 2002	Saskatchewan	TS	M	Outpatient	30 years	Fever, cough
52	17 January 2002	Saskatchewan	NPS	F	Outpatient	72 years	Fever, sore throat
57	18 January 2002	Saskatchewan	NPS	F	Outpatient	37 years	Fever, rhinitis, cough
140	6 March 2002	Nova Scotia	NPA	M	NA	NA	NA
146	20 February 2002	Nova Scotia	NA	F	NA	67 years	NA
214	27 February 2002	Manitoba	NPS	F	Hospitalized	2 years	Nonproductive cough, hoarse voice
435	9 March 2002	Manitoba	NPS	M	Outpatient	6 months	Bronchiolitis
449	1 March 2002	Manitoba	NPS	M	Hospitalized	7 months	Cough, coryza, lower-respiratory-tract infection
470[a]	12 March 2002	Manitoba	TS	M	Hospitalized	83 years	Colon cancer with lung metastases, sore throat, fever, raspy voice, congestion, body aches, myalgia, phlegm in throat, chronic obstructive pulmonary disease
483[a]	12 March 2002	Manitoba	TS	F	Outpatient	74 years	Sore throat, myalgia, congestion
495	12 March 2002	Manitoba	LA	M	Outpatient	4 months	None noted (sudden death due to smothering by bed linens; chronic laryngotracheobronchitis noted on autopsy)
522	20 March 2002	Manitoba	NPA	M	Outpatient	3 months	NA
525[a]	8 March 2002	Manitoba	PS	F	Outpatient	86 years	Fever, malaise, sore throat, cough, sputum
527[a]	8 March 2002	Manitoba	PS	M	Outpatient	95 years	Fever, malaise, cough, sputum, shortness of breath
528[a]	8 March 2002	Manitoba	PS	M	Outpatient	77 years	Fever, malaise, cough, sputum
531[a]	8 March 2002	Manitoba	PS	M	Outpatient	92 years	Fever, malaise, cough, sputum, pleuritic chest pain, shortness of breath (died 5 days after onset of symptoms)
535[a]	8 March 2002	Manitoba	PS	M	Outpatient	100 years	Fever, cough, sputum
543	31 March 2002	Manitoba	NPA	M	Hospitalized	1 month	Bronchiolitis

NOTE. LA, lung autopsy; NA, data not available; NPA, nasopharyngeal aspiration; NPS, nasopharygeal swab; PS, pharyngeal swab; TS, throat swab.

[a] Specimens collected during an outbreak of acute respiratory-tract infection in a personal-care home.

contained 2 μL of QIAGEN OneStep RT-PCR enzyme mix, 10 μL of 5× QIAGEN OneStep RT-PCR buffer, 400 μmol/L dNTP, 0.6 μmol/L of each primer, and 10 μL of Q-solution, in a final volume of 50 μL. The thermocycler conditions used were 30 min at 50°C, for reverse transcription; 15 min at 95°C, for the activation of the HotStart DNA polymerase; 35 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C; and an extension for 10 min at 72°C. Five microliters of the RT-PCR reaction was added to the nested PCR mixture, which contained 5 μL of 10× *Taq* polymerase buffer, 200 μmol/L dNTP, 0.5 μmol/L of each primer, and 5 U of *Taq* polymerase (Sigma). The thermocycler conditions used for the nested PCR were 5 min at 95°C; 40 cycles of 1 min at 95°C, 1 min at 55°C, and 2 min at 72°C; and an extension for 10 min at 72°C. The PCR products were purified by use of a QIAquick PCR purification kit (QIAGEN) and were sequenced on an ABI 377 Sequencer, by use of a fluorescent dye–terminator kit (Applied Biosystems). The DNA sequences were assembled and analyzed with the Seqman, Editseq, and Megalign programs in the Lasergene suite (DNASTAR). Phylogenetic trees were generated by the neighbor-joining method using the MEGA program [9].

To monitor the efficiency of nucleic-acid extraction, each sample was also tested for the presence of the RNase P housekeeping gene by use of a 1-step RT-PCR kit under the following conditions: 30 min at 50°C, for reverse transcription; 15 min at 95°C, for the activation of the HotStart DNA polymerase; 50 cycles of 15 s at 94°C, 30 s at 50°C, and 30 s at 72°C; and an extension for 7 min at 72°C. Of the 525 specimens tested, all were positive for the RNase P gene.

The HCoV-NL63 sequences described in the present study have been deposited in GenBank, under accession numbers AY675541–AY675553.

Results. The National Microbiology Laboratory received a total of 525 specimens collected from patients with ARI, from provincial public health laboratories in Manitoba (377 specimens), Saskatchewan (104 specimens), and Nova Scotia (44 specimens). These representative specimens were selected to be negative for (1) influenza viruses A and B; (2) parainfluenza virus (PIV) 1, 2, and 3; (3) adenovirus; and (4) RSV, by direct or indirect fluorescence assays and/or virus isolation, and to be negative for hMPV, by RT-PCR. The specimens were collected during January–April 2001 (185 specimens), October 2001 (30 specimens), and January 2002–April 2002 (302 specimens); the collection date for 8 specimens was unknown. Specimens analyzed included 276 samples from throat swabs, 92 from nasopharyngeal swabs, 72 from nasal aspirations, 20 from nasal swabs, 10 from lung-tissue biopsies, 17 from tracheal aspirations, 5 from bronchoalveolar lavages, 8 from pharyngeal

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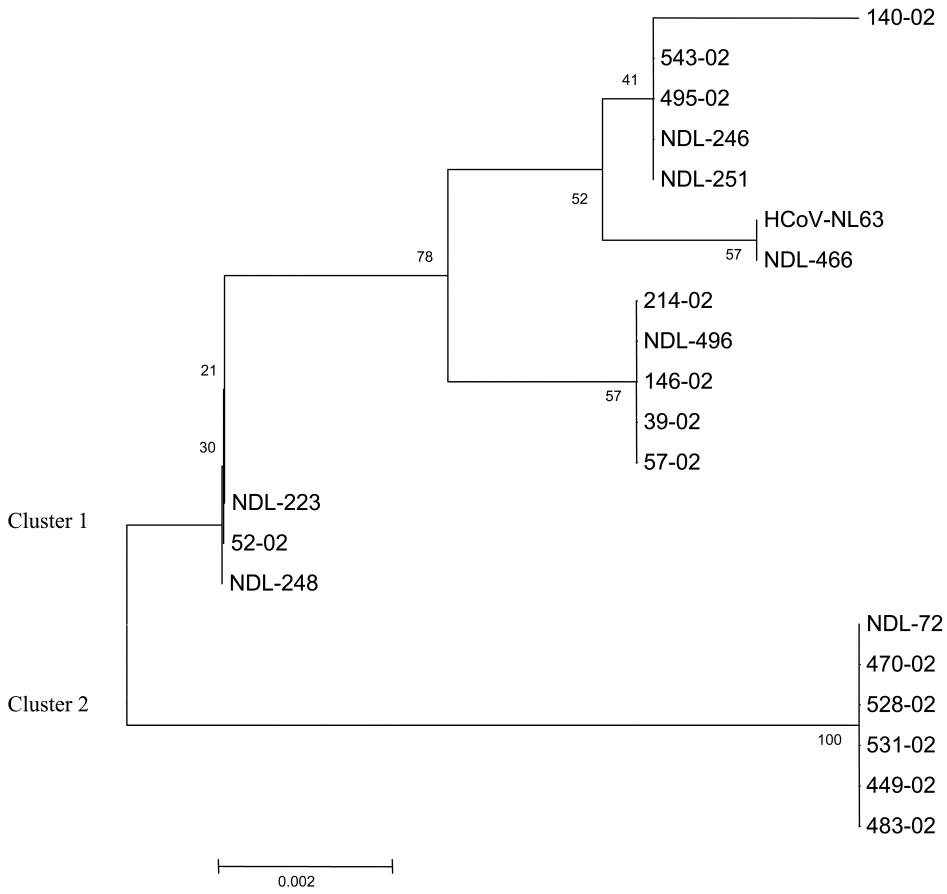

Figure 1. Phylogenetic analysis of human coronavirus NL 63 (HCoV-NL63) isolates. Genetic sequences were determined for nucleotides 5856–6280 of the 1a gene. The corresponding sequences from previously reported Dutch HCoV-NL63 isolates were also included. Phylogenetic analysis was performed by use of the neighbor-joining method of the MEGA program. The Dutch reference strain is designated by "HCoV-NL63," and the Dutch isolates are designated by "NDL" followed by an isolate number (i.e., NDL-246). The isolates detected in the present study are designated in terms of specimen number and the (2-digit) year of detection (i.e., 140-02).

swabs, 3 from throat washes, and 22 other, miscellaneous specimens from the respiratory system.

Of the 525 specimens tested, 19 (3.6%) were positive, by RT-PCR, for HCoV-NL63, and they were from all 3 provinces: 13 (68%) from Manitoba, 4 (21%) from Saskatchewan, and 2 (11%) from Nova Scotia. HCoV-NL63 activity was found during January (4 specimens [21%]), February (2 specimens [11%]), and March (13 specimens [68%]) 2002 and subsided during late spring; in contrast, no specimens positive for HCoV-NL63 were retrieved for the same period during 2001. The sex distribution of these 19 specimens was 68% (13) male and 32% (6) female (table 1). Patients with HCoV-NL63 were 1 month–100 years old (median age, 37 years). The proportion of positive specimens was significantly higher in the 0–5-year-old age group than in the >5-year-old age group (table 1) (8/110 vs. 11/402; $P = .05$). Seven patients from the >50-year-old age group were involved in an outbreak of ARI in a personal-care home in Manitoba (table 1). HCoV-NL63 was found in 7 of the 8 patients from this outbreak whose samples were analyzed in this study.

For 16 of the 19 patients positive for HCoV-NL63, information on symptoms was available from medical files; the main clinical presentations reported were fever (15 patients [79%]), sore throat (5 patients [26%]), and cough (9 patients [47%]) (table 1). Other observed symptoms included bronchiolitis (2 patients), rhinitis (1 patient), and lymphadenitis (1 patient), and 2 patients died—1 because of unrelated causes (this patient, a male infant, was smothered) (table 1). Of the 4 patients (21%) with HCoV-NL63 who were hospitalized, 3 (75%) were in the 0–5-year-old age group, and 1 was in the >50-year-old age group (table 1).

For 13 of the 19 specimens positive for HCoV-NL63, nucleotide sequences were determined for nucleotides 5856–6280 of the 1a gene. Comparison of these sequences with those published for Dutch isolates of HCoV-NL63 showed that the 1a genes were relatively well conserved, with 98.1%–100% nucleic-acid identity between specimens. The phylogenetic tree of the HCoV-NL63 isolates showed the existence of 2 major groups or clusters that contained both Canadian and Dutch strains of HCoV-NL63 (figure 1). Similar Canadian HCoV-NL63 strains

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were isolated from adults and children in all 3 provinces and during different outbreaks. Strains isolated from the outbreak of ARI in a personal-care home in Manitoba were grouped together into cluster 2.

Discussion. Although, in a study like this one, the inclusion of a control group of healthy individuals is necessary to clearly demonstrate a causal relationship, the detection of HCoV-NL63 in respiratory-tract specimens from patients suffering from ARI of unknown causes strongly suggests that it is associated with respiratory illness. This finding supports the association, found by van der Hoek et al. [2], of HCoV-NL63 with ARI. It also demonstrates for the first time that HCoV-NL63 was present in several Canadian provinces during the 2002 winter season, suggesting that HCoV-NL63 may be circulating worldwide. We detected the presence of HCoV-NL63 in 19 (3.6%) of the 525 analyzed specimens that were negative for (1) influenza viruses A and B; (2) PIV 1, 2, and 3; (3) adenovirus; (4) RSV; and (5) hMPV; and these results provide further evidence of the contribution of HCoV-NL63 to ARI—and of the significant burden that it therefore may present to health-care systems. For the time period that was analyzed in the present study, peak activity of HCoV-NL63 was found to occur during January–March 2002. Although sampling occurred only during the winter season (October–April), our results are consistent with van der Hoek et al.'s [2] finding that HCoV-NL63 appears to be transmitted predominantly during the winter season. Other HCoVs, as well as influenza and RSV, which are involved in a substantial number of hospitalizations for ARI, were also shown to be present in patients with ARI during that time of the year [7]. The clinical symptoms associated with HCoV-NL63 infection are also comparable to those observed to be associated with influenza, RSV, and other HCoVs, making it impossible, at least on the basis of seasonality and clinical manifestations, to differentiate between these viral infections. Previous estimates of the contributions of RSV and other etiological agents to ARI based on these parameters may have been biased because the involvement of other new respiratory viruses such as hMPV and HCoV-NL63 were overlooked [10]. Thus, the systematic detection of HCoV-NL63 in respiratory specimens may improve the understanding of the etiology of ARI; however, the possibility of dual infection cannot be excluded, because the present study utilized samples from patients with ARI who were tested—and found to be negative—for only (1) influenza viruses A and B; (2) PIV 1, 2, and 3; (3) adenovirus; (4) RSV; and (5) hMPV. Nor can it be ruled out that HCoV-NL63 can exist asymptomatically in an individual, because samples from healthy individuals were not included. In addition, the present study provides evidence that HCoV-NL63 may have been associated with an outbreak of ARI in a personal-care home. All hospitalizations occurred in the 0–5-year-old and >50-year-old age groups, which suggests that, like the other HCoVs and RSV, HCoV-NL63 may cause more-severe ARI in frail patients, such as infants and the elderly, than in other patients [4, 6, 11–13].

Phylogenetic analysis based on the 1a gene confirms previously published results and shows the presence of viruses with different molecular markers [2]. The increased number of isolates detected provides further evidence of genetic diversity and the presence of 2 genetic clusters. Close clustering of HCoV-NL63 isolates recovered from different Canadian provinces and from The Netherlands suggests that the evolutionary pattern of HCoV-NL63 does not presently correlate with geographic location.

In summary, our data suggest that HCoV-NL63 may play a significant role in ARI, especially in young children and the elderly. More-comprehensive studies, which would include data on prevalence, risk factors, and use of health services, are necessary to determine both the importance of HCoV-NL63 in ARI and its impact on the health-care system.

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