MAJOR ARTICLE

Evidence of a Novel Human Coronavirus That Is Associated with Respiratory Tract Disease in Infants and Young Children

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(See the brief report by Esper et al., on pages 499–502, and the editorial commentary by McIntosh, on pages 489–91.)

Background. The etiological agents responsible for a substantial proportion of respiratory tract diseases have not been identified. We sought to determine whether novel human coronaviruses (HCoVs) are circulating in New Haven, Connecticut, and, if so, whether they are associated with respiratory tract disease in infants and young children.

Methods. We developed a polymerase chain reaction (PCR)–based approach for screening specimens from the respiratory tracts of symptomatic children. PCR probes that target regions of the replicase 1a gene that are conserved among genetically diverse animal CoVs and HCoVs were designed. Using these probes, we identified genomic sequences of a novel HCoV, designated "New Haven coronavirus" (HCoV-NH). Thereafter, we designed specific probes to screen respiratory specimens from children <5 years old for this novel HCoV. Clinical features associated with HCoV-NH infection were identified.

Results. Seventy-nine (8.8%) of 895 children tested positive for HCoV-NH. Cough, rhinorrhea, tachypnea, fever, abnormal breath sounds, and hypoxia were the most common findings associated with HCoV-NH infection. Sequence analysis revealed that HCoV-NH is closely related to a novel HCoV recently reported in The Netherlands.

Conclusions. The novel HCoVs identified in New Haven and The Netherlands are similar and likely represent the same species. This newly discovered virus may have worldwide distribution and may account for a significant proportion of respiratory tract disease in infants and young children.

Infections of the respiratory tract are a leading cause of morbidity and mortality worldwide [1]. However, for a substantial proportion of these infections, the agents that are causing the disease remain undetermined [2–4]. Recently, there has been renewed interest in human coronaviruses (HCoVs), in part because of the identification of a novel CoV associated with severe acute respiratory syndrome (SARS) [5–7]. Before the emergence of SARS-CoV, HCoVs were generally thought to cause mild, self-limited infections of the upper respiratory tract [8]. However, it is now known that HCoVs can cause severe disease in immunocompromised individuals [9]. The study of HCoVs has been hampered by the difficulty in propagating these viruses in vitro and the lack of specific diagnostic tests with which to identify potentially novel viruses. Therefore, the possibility exists that unidentified HCoVs are the cause of some proportion of the respiratory tract diseases for which etiological agents have not been identified.

To determine whether novel HCoVs are circulating and, if so, whether they are responsible for respiratory tract disease in children, we developed a strategy to screen for previously unknown HCoVs. Our initial assumption was that all CoVs must have conserved functions and that these conserved functions are reflected in the genome. The replicase of CoVs is an RNA-dependent RNA polymerase, the function of which is not provided by the host cell and, therefore, must be evolu-

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tionarily maintained by all CoVs. Therefore, we designed polymerase chain reaction (PCR) probes that target regions of the CoV replicase 1a gene that are conserved among HCoVs, avian CoVs, and mammalian CoVs. Using this approach, we identified genetic evidence of a novel HCoV circulating in New Haven, CT. Probes specific for this HCoV, designated "New Haven coronavirus" (HCoV-NH), were then used to screen specimens from the respiratory tracts of symptomatic children. During our screening of respiratory specimens for this HCoV, 2 studies from The Netherlands reported the identification of a novel HCoV [10, 11]. This virus is a group 1 CoV and is most closely related to HCoV-229E and transmissible gastroenteritis virus (TGEV), a virus of pigs. Comparisons of the sequences of the HCoV identified in The Netherlands and HCoV-NH revealed that these viruses are closely related and likely represent the same species. Here, we describe the seasonal distribution and clinical manifestation of disease associated with HCoV-NH infection.

PATIENTS, MATERIALS, AND METHODS

Primer design and reverse transcriptase (RT)–PCR screening. Primers for the detection of CoVs were based on conserved regions of the replicase 1a gene of groups 1, 2, and 3 CoVs and SARS-CoV. The replicase 1a genes from avian infectious bronchitis virus (GenBank accession number AJ311317), bovine CoV (BCoV) strain LUN (GenBank accession number AF391542), BCoV strain Quebec (GenBank accession number AF220295), BCoV isolate BCoV-ENT (GenBank accession number AF391541), HCoV-229E (GenBank accession number NC_002645), TGEV (GenBank accession number NC_002306), and SARS-CoV TOR2 (GenBank accession number AY274119) were aligned by use of Clustal W in the Lasergene software package (version 5.05; DNASTAR). Two conserved regions were identified, and primers corresponding to these regions were synthesized (Yale Oligonucleotide Laboratory, Department of Pathology, New Haven, CT). The forward primer, 5′-GCGCAAAATAATGAATTAATGCC-3′ (underscoring indicates a G/C clamp), and the reverse primer, 5′-GACGCACCACCATATGAATCCTG-3′, represent consensus sequences of conserved regions within the 3′ 1000 bases of the replicase 1a gene of all of the CoVs listed above (relative to sequences 11,781–12,285 of HCoV-229E). The predicted length of the amplicons produced by these primers is ~550 bp. RNA from respiratory specimens obtained from the Clinical Virology Laboratory, Yale–New Haven Hospital, was extracted by use of the QIAamp Viral RNA Mini Kit (Qiagen), in accordance with the manufacturer's instructions. cDNA for each respiratory specimen was synthesized with random hexamer primers and MuMLV RT (New England Biolabs), in accordance with the manufacturer's instructions. cDNAs were subsequently screened by PCR with HotStar *Taq* polymerase (Qiagen), in accordance with the manufacturer's instructions. The following amplification program was used: 15 min at 95°C; 40 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C; and a final extension of 10 min at 72°C. For the initial screening of respiratory specimens, RNA was extracted from MRC-5 cells infected with HCoV-229E (ATCC VR-740) as a positive control. Each set of RT-PCR amplifications included appropriate negative controls. PCR products were analyzed by agarose gel electrophoresis. After the initial screening of respiratory specimens, amplicons of the predicted molecular weight were isolated and sequenced. Sequencing was performed by use of Applied Biosystems 377 DNA automated sequencers (W. M. Keck Biotechnology Resource Lab, Yale University School of Medicine). Sequences that corresponded to a potential novel HCoV were identified, and primers specific for this agent were synthesized. The forward primer, 5′-GCGCTATGAGGGTGGTTGTAAC-3′, and the reverse primer, 5′-CGCGCAGTTAAAAGTCCAGAATTAAC-3′, amplify a 215-bp region of the novel HCoV genome. These primers target regions of the novel HCoV genome that are distinct relative to the corresponding region of the HCoV-229E genome. Screening by PCR with these primers was performed by use of the following amplification program: 15 min at 95°C; 40 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and a final extension of 10 min at 72°C. These primers were used to screen pooled respiratory specimens.

Respiratory specimens. We chose to screen specimens that were obtained from the respiratory tracts of symptomatic children <5 years old and that tested negative for respiratory syncytial virus (RSV), influenza viruses A and B, human parainfluenza viruses 1–3, and adenovirus by direct fluorescent antibody assay (DFA). These specimens, which were obtained from both ambulatory and hospitalized patients, were screened for presence of human metapneumovirus (hMPV) by use of an RT-PCR–based approach described elsewhere [12, 13] and were submitted to the Clinical Virology Laboratory, Yale–New Haven Hospital, for diagnostic testing. Specimens were obtained from 1 January 2002 to 14 February 2003. Children could be counted more than once if the specimens were obtained >30 days apart. Clinical data from the children who tested positive for HCoV-NH were obtained by extracting information from medical records and recording the data on a standardized form. Children who had evidence of infection with another viral respiratory pathogen were excluded when the clinical features associated with HCoV-NH infection were tabulated. Comorbidity was defined as prematurity (gestational age of <35 weeks), underlying pulmonary disease, genetic syndromes, acquired immunosuppression, malignancies, and congenital heart disease. The Yale University Human Investigations Committee approved the collection and screening of respiratory specimens.

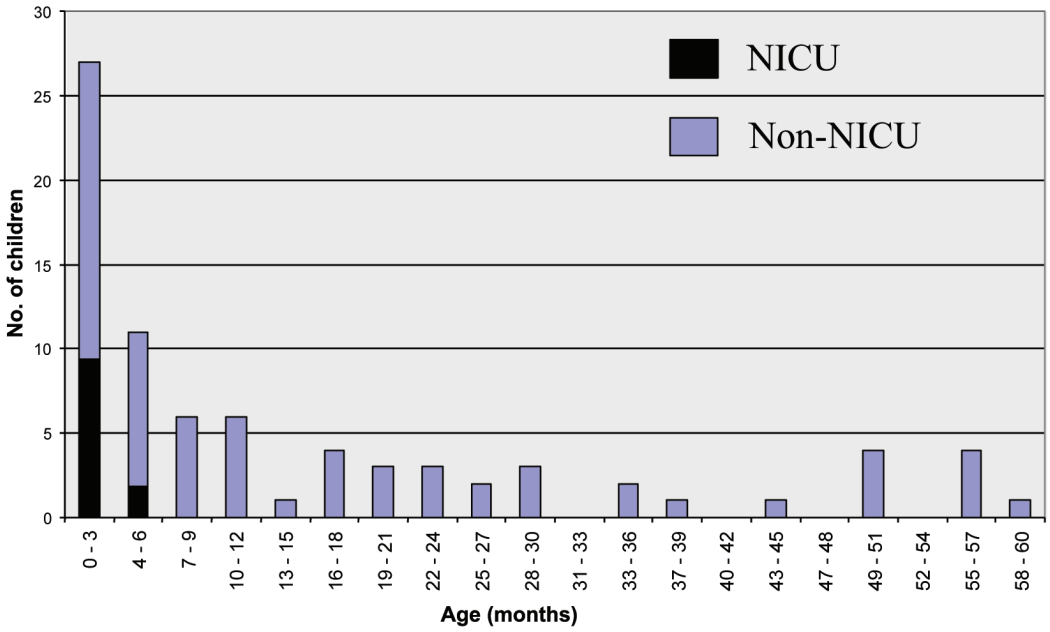

Figure 1. Age distribution of New Haven coronavirus (HCoV-NH)–positive children <5 years old. HCoV-NH infection in children hospitalized since birth at the newborn intensive care unit (NICU), Yale–New Haven Hospital (CT), is represented by black bars.

Sequencing and phylogenetic analysis. The amplicon of each specimen that tested positive by RT-PCR was sequenced to confirm the presence of HCoV-NH. Phylogenetic analysis was performed by use of the Lasergene software package described above.

RESULTS

Identification of a novel CoV sequence. Using the primers that target the conserved regions of the CoV replicase 1a gene, we screened 601 respiratory specimens for CoVs. The screening reaction was performed on pooled RNA; each pooled amplification reaction included 5–10 individual specimens. Of the 80 pooled amplification reactions, 17 yielded an amplicon of ~550 bp (data not shown). After these amplicons were sequenced, a nucleotide BLAST (available at: http://www.ncbi.nlm.nih.gov/BLAST/) search was performed. HCoV-OC43 was identified in 8 pooled reaction products, and HCoV-229E was identified in 1 pooled reaction product. Six amplicons either were human DNA or did not yield an interpretable sequence. The 2 remaining amplicons were similar and represented novel sequences most closely related to group 1 CoVs. These sequences were ~69%–71% identical to HCoV-229E and TGEV on the nucleotide level and ~68% identical to HCoV-229E and TGEV on the amino acid level. PCR primers specific for these novel sequences of HCoV-NH were then synthesized, and these primers were used to screen all respiratory specimens thereafter.

Screening of respiratory specimens for HCoV-NH. In total, 1265 respiratory specimens from 895 children were screened by RT-PCR with primers specific for HCoV-NH (the 601 specimens from the initial screening were rescreened by RT-PCR with these primers). Screening reactions were again pooled, with 5–10 individual specimens in each pool. If a pooled reaction produced an appropriately sized amplicon, the specimens in that pool were individually tested. Seventy-nine (8.8%) of these individual specimens were found to be positive for HCoV-NH. Two children had 2 positive specimens each; the specimens were obtained 5 days apart for 1 child and 7 days apart for the other child. In both instances, the 2 positive specimens were considered to be the result of a single episode of HCoV-NH infection. The median age of the 79 HCoV-NH–positive children was 6.5 months. Fifty (63.3%) were <1 year old, and 27 (34.2%) were 0–3 months old. Forty-nine (62.0%) were male. Eleven of the HCoV-NH–positive children had been hospitalized since birth at the newborn intensive care unit (NICU), Yale–New Haven Hospital. The age distribution of the HCoV-NH–positive children is shown in figure 1.

Clinical features associated with HCoV-NH infection. Clinical data were available for 76 of the 79 HCoV-NH–positive children. Of these 76 children, 9 (11.8%) had evidence of recent infection with another respiratory virus—2 were coinfected with hMPV, and 7 were found to be coinfected with another respiratory virus by DFA on other respiratory specimens that had been collected during the same hospitalization or illness (1 had a parainfluenza infection, and 6 had an RSV infection). Among the 67 children who tested positive for HCoV-NH only, cough, rhinorrhea, tachypnea, fever, abnormal breath sounds, and hypoxia were the most common findings (table 1). Thirty-five (52.2%) of these 67 children had an underlying comor-

Table 1. Clinical features associated with New Haven coronavirus infection in children <5 years old.

Clinical feature	NICU children (n = 11)	Non-NICU children (n = 56)	Total (n = 67)
Cough	0 (0)	43 (76.8)	43 (64.2)
Rhinorrhea	3 (27.3)	38 (67.9)	41 (61.2)
Tachypnea[a]	9 (81.8)	30 (53.6)	39 (58.2)
Fever[b]	2 (18.2)	30 (53.6)	32 (47.8)
Rhonchi, rales, or abnormal breath sounds	6 (54.5)	24 (42.9)	30 (44.8)
Hypoxia[c]	9 (81.8)	16 (28.6)	25 (37.3)
Chest retractions	3 (27.3)	19 (33.9)	22 (32.8)
Wheezing	1 (9.1)	20 (35.7)	21 (31.3)
Stridor	0 (0)	4 (7.1)	4 (6.0)
Abnormal chest-radiograph findings[d]	5/7 (71.4)	20/31 (64.5)	25/38 (65.8)

NOTE. Data are no. (%) of children, except where noted. NICU, newborn intensive care unit.

[a] On the basis of normal values for age-specific respiratory rates.

[b] Temperature >38°C.

[c] Oxygen saturation ⩽90%.

[d] Data are no. (%) of abnormal chest-radiograph findings per the no. of chest radiographs obtained.

bidity (19 [28.4%] were born prematurely). Of the 38 children for whom a chest radiograph was obtained, 25 (66.0%) had abnormal findings, which were characterized by peribronchial cuffing, atelectasis, and/or infiltrates.

The clinical features associated with HCoV-NH infection among the 11 children hospitalized since birth at the NICU are shown in table 1. The median age of these children at the time their positive specimens were obtained was 26 days (range, 1–151 days). The age distribution and the time of infection of the HCoV-NH–positive children hospitalized since birth at the NICU are shown in figures 1 and 2, respectively. Five of the 11 children were infected during February 2002. The hospitalization of all of these children overlapped. Three of the 11 children tested positive for HCoV-NH during a 3-week period spanning late January and early February 2003; these 3 children were hospitalized at the NICU at the same time.

Two of the 79 HCoV-NH–positive children died; both had been hospitalized since birth at the NICU. One child had been diagnosed with hydrops fetalis in utero and died on day 2 of life, 1 day after a respiratory specimen had been collected that tested positive for HCoV-NH. The second child, who had been born at 28 weeks gestation, died on day 170 of life; a respiratory specimen collected on day 151 of life tested positive for HCoV-NH. This child also had a history of necrotizing enterocolitis and liver failure before day 151 of life and ultimately died of multiorgan system failure.

Seasonal distribution of HCoV-NH infection. The weekly distribution of HCoV-NH–positive children is shown in figure 2, and the percentage of positive specimens detected per month is shown in table 2. Of the specimens obtained from 1 January 2002 to 31 December 2002, a majority (42/67 [62.7%]) that tested positive were obtained during the first 10 weeks of the year. Relatively few positive specimens (13/67 [19.4%]) were obtained from June to late November.

Phylogenetic analysis of HCoV-NH. Sequence analysis was performed on the amplicon derived from each positive specimen (GenBank accession numbers AY870943–AY871008). Phylogenetic analysis of a 126-bp portion of the HCoV-NH–specific primer region, which contains representative sequences of HCoV-NH, is shown in figure 3. Overall, the amplified region of the putative replicase 1a gene of HCoV-NH was highly conserved. These sequences closely matched the sequences of the replicase 1a gene of the novel HCoV recently identified in The Netherlands [10, 11]. Several distinct polymorphisms were identified among the HCoV-NH isolates; these polymorphisms were not present in the HCoV identified in The Netherlands. Most of the polymorphisms in the replicase 1a gene among HCoV-NH isolates did not change the predicted amino acid sequence (data not shown).

DISCUSSION

Using a PCR-based approach to screen for previously unknown HCoVs, we identified evidence of the existence of a novel HCoV, and we have described the clinical features associated with infection with this agent. Our approach consisted of identifying conserved regions of the replicase 1a gene of a variety of animal CoVs and HCoVs and then exploiting the conserved genomic domains to identify a previously unknown HCoV. This approach is based entirely on knowledge of CoV genomes, and so identification of a CoV does not require the ability of the virus to propagate in vitro or in animal models. This type of genome-based screening has been used previously to identify human bacterial pathogens by targeting conserved elements of rRNA [14]. After the observation of CoV-like particles in re-

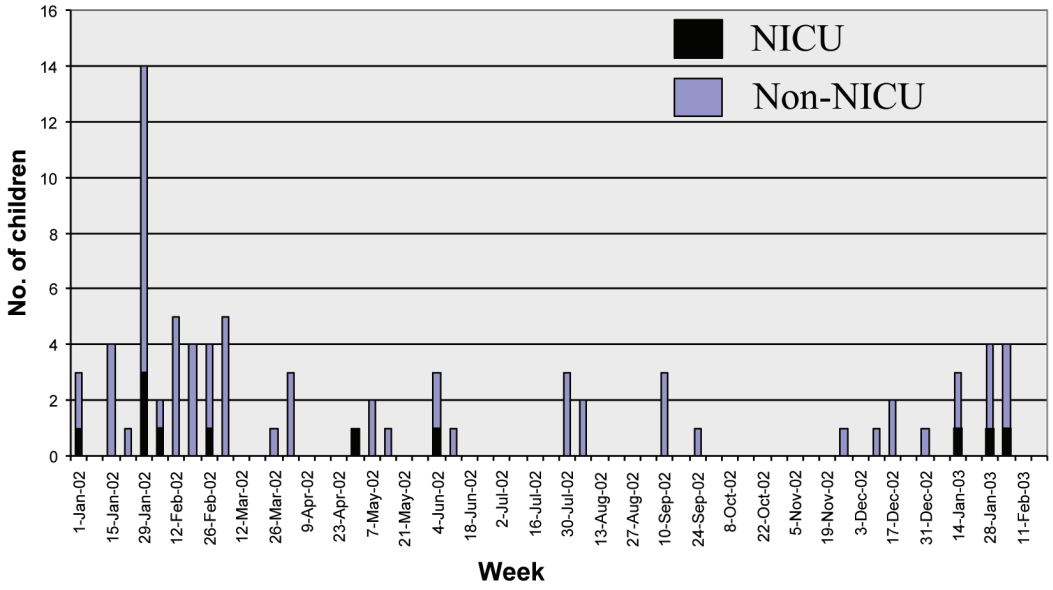

Figure 2. Weekly distribution of New Haven coronavirus (HCoV-NH)–positive children <5 years old from January 2002 to mid-February 2003. For clarity, only the first day of every other week is labeled. HCoV-NH infection in children hospitalized since birth at the newborn intensive care unit (NICU), Yale–New Haven Hospital (CT), is represented by black bars.

spiratory secretions from individuals with SARS, broadly reactive PCR was used to identify the pathogen; unlike in the present study, in which the replicase 1a gene was targeted, the replicase 1b gene was targeted in the identification of the agent that causes SARS [5].

In the present study, the initial set of primers, used to screen pooled specimens, were less sensitive than the HCoV-NH–specific primers. Many of the respiratory specimens in the initial pool of 601 specimens that tested negative by PCR with the replicase 1a gene primers tested positive by PCR with the HCoV-NH–specific primers. Subsequent sequence analysis of the target sites for the replicase 1a gene of HCoV-NH (data not shown) revealed several mismatches between the genome and primer sequence. This finding likely accounts for the relative decreased sensitivity of the replicase 1a gene primers. Among the 601 specimens initially screened, the 2 pooled reactions that tested positive for HCoV-NH contained multiple positive specimens. This may account for why these pooled specimens tested positive. It is also unclear why primers constructed for the detection of the 2 common HCoVs, HCoV-229E and HCoV-OC43, would not have detected HCoV-NH. Presumably, the genomic sequence targeted for the detection of HCoV-229E and HCoV-OC43 is specific for these viruses. The sensitivity of the CoV consensus primers and the HCoV-NH–specific primers is unknown. Determination of the sensitivity will require propagation of the virus and development of a method for quantitating the number of viral particles (such as an infectivity assay or a method based on electron microscopy, neither of which have been completely developed for HCoV-NH). As further genetic data become available, more-sensitive primers may be developed.

Our approach complements that of traditional virology, in which viruses are identified by their effects on cells in tissue culture. However, the identification of previously unknown viruses on the basis of in vitro findings has significant shortcomings, not the least of which is the inability to amplify and characterize unique genetic sequences. This obstacle was overcome by Fouchier et al. [10] and van der Hoek et al. [11], who used the tools of molecular biology to identify and sequence the genome of a novel HCoV in The Netherlands after propagation in cell culture. hMPV was discovered by use of a similar approach that was based on in vitro virus propagation and genomic amplification techniques [15]. Like the 2 groups from The Netherlands, we have evidence that this novel HCoV can be propagated in cell culture. The results of RT-PCR assays of passaged cell culture supernatants suggested the growth of HCoV-NH in vitro (data not shown).

There were early hints that HCoV-229E and HCoV-OC43 were not the only common HCoVs. The first HCoV isolated, B814, was not serologically related to either HCoV-229E or HCoV-OC43 [16]. McIntosh et al. demonstrated that 3 of the 6 OC strains isolated from adults with colds appeared to be serologically unrelated, or at least distantly related, to HCoV-OC43 and HCoV-229E [17]. Experimental infection of human volunteers with these 6 OC strains confirmed this finding [18]. It was, therefore, unlikely that HCoV-229E and HCoV-OC43 were the only HCoVs. The viral pathogen newly identified in New Haven

Table 2. Respiratory specimens from children <5 years old tested for New Haven coronavirus (HCoV-NH) from January 2002 to mid-February 2003.

Year, month	No. tested		No. (%) of HCoV-NH–positive children
	Specimens	Children	
2002			
January	148	99	8 (8.1)
February	117	81	27 (33.3)
March	123	98	8 (8.2)
April	74	51	3 (5.9)
May	48	32	4 (12.5)
June	19	15	4 (26.7)
July	42	17	0 (0)
August	29	19	5 (26.3)
September	38	22	4 (18.2)
October	89	61	0 (0)
November	137	105	0 (0)
December	144	104	4 (3.8)
2003			
January	177	129	4 (3.1)
February	80	62	8 (12.9)
Total	1265	895	79 (8.8)

and The Netherlands may represent the first of many antigenically distinct groups of HCoVs to be characterized.

In the present study, HCoV-NH was associated with both upper and lower respiratory tract disease in infants and young children. Because ours was not a population-based study and because a control group was not included, it is impossible to determine the spectrum of disease caused by HCoV-NH infection. Our study screened specimens submitted to a diagnostic laboratory only; therefore, the proportion of children with clinical illness reported here likely does not reflect that of the general population. Likewise, the issue of causality remains unresolved. Prospective population-based studies are required. Nonetheless, the identification of genetic sequences of this HCoV from a large number of children with respiratory tract illnesses strongly suggests that HCoV-NH infection plays a role in disease. Our data suggest that HCoV-NH, like other HCoVs, may have a seasonal distribution [8]. Animal CoVs cause disease in organ systems other than the respiratory tract, such as the gastrointestinal system and the central nervous system [19]. SARS-CoV is excreted in the stool of infected individuals [6]. Therefore, it is necessary to determine whether HCoV-NH is present in other body fluids and whether it is associated with other clinical syndromes.

The percentage of children who tested positive for this novel HCoV in our study (8.8%) was higher than the percentages of patients who tested positive in the studies by van der Hoek et al. (1.6%) and Fouchier et al. (2.9%) [10, 11]. The reasons for this are unclear. Both our study and those in The Netherlands screened respiratory specimens submitted to a diagnostic virology laboratory and used a PCR–based approach for screening. The characteristics of the study populations and the time of the acquisition of the specimens screened may explain the differences in the observed percentages of positive subjects between our study and the 2 studies in The Netherlands. Fouchier et al. limited screening for the novel HCoV to 3 months during late autumn and early winter [10] and, therefore, may have missed the peak circulation of the virus in the population. Also, we chose to screen only children <5 years old, whereas van der Hoek et al. chose to screen individuals of all ages [11]. Similar to other respiratory viruses, symptomatic infection with this newly discovered HCoV may be more common during childhood; therefore, we may have been more likely to detect a higher percentage of infected subjects in the present study. HCoVs can cause severe disease in infants [20]. In the present study, a majority (50/79 [63.3%]) of HCoV-NH–positive children were, in fact, <1 year old, and 34.2% were ≤3 months old.

Eleven (13.9%) of the 79 HCoV-NH–positive children in the present study had been hospitalized since birth at the NICU.

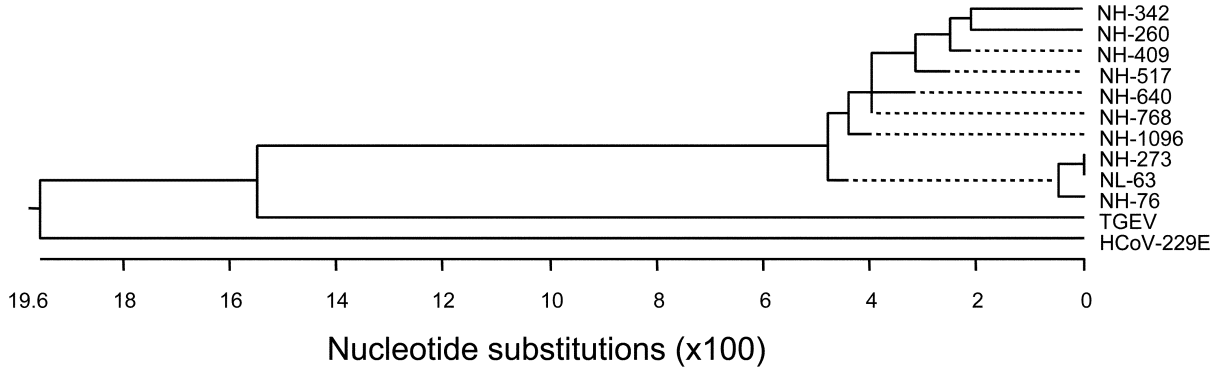

Figure 3. Phylogenetic analysis of New Haven coronavirus (HCoV-NH). To construct the phylogenetic tree, sequences of a 126-bp portion of the replicase 1a gene of a representative sample of HCoV-NH (NH) amplicons, the HCoV recently identified in The Netherlands (NL-63), HCoV-229E, and transmissible gastroenteritis virus (TGEV) were used.

There were 2 apparent temporal clusters of HCoV-NH infection at the NICU, during February 2002 and during January and February 2003. Although these may represent nosocomial outbreaks, other modes of infection, such as perinatal transmission, cannot be discounted. HCoV-NH was detected in a child diagnosed with hydrops fetalis. Whether this represents congenital infection with HCoV-NH remains to be determined.

In conclusion, the present results demonstrate the power of the tools of molecular biology to define and characterize potential infectious agents associated with human disease. The novel HCoV identified in New Haven and The Netherlands was associated with both upper and lower respiratory tract disease in infants and young children. Whether this virus is associated with other clinical syndromes remains to be determined. Population-based studies are required to define the burden of disease caused by this novel HCoV, and such studies could provide information on causality.

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