JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2009, p. 3486–3492 Vol. 47, No. 11
0095-1137/09/$12.00 doi:10.1128/JCM.00832-09

Epidemiology and Clinical Presentations of Human Coronavirus NL63 Infections in Hong Kong Children▽†

Ting Fan Leung,[1]* Chung Yi Li,[1] Wai Yip Lam,[2] Gary W. K. Wong,[1] Edmund Cheuk,[2] Margaret Ip,[2] Pak Cheung Ng,[1] and Paul K. S. Chan[2]

Departments of Paediatrics[1] and Microbiology,[2] The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong

Received 26 April 2009/Returned for modification 2 June 2009/Accepted 3 September 2009

Human coronavirus NL63 (HCoV-NL63) has been found in children presenting with respiratory tract infections (RTIs). However, the epidemiology and clinical course of this newly identified virus have not been fully elucidated. This study investigated the epidemiology, seasonality, and clinical features of HCoV-NL63 in Hong Kong children. This study consisted of two cohorts of children hospitalized in a university-affiliated teaching hospital. In the 12-month retrospective part of the study, reverse transcription-PCR was used to detect HCoVs in nasopharyngeal aspirates (NPAs). Positive samples were sequenced to confirm the identity of the virus and to determine its phylogenetic relationship with the HCoV-NL63 strains found elsewhere. The second part covered a subsequent 12-month period in which patients were prospectively recruited. Altogether, 1,981 and 1,001 NPA specimens were studied in 2005–2006 and 2006–2007, respectively. Seventy-four (2.5%) HCoV isolates were identified and consisted of 17 (0.6%) HCoV-NL63 isolates, 37 (1.2%) HCoV-OC43 isolates, 14 (0.5%) HCoV-HKU1 isolates, and 6 (0.2%) HCoV-229E isolates. HCoV-NL63 infection was more common in 2006–2007 than 2005–2006 (1.2% and 0.3%, respectively; $P = 0.006$). From 2005 to 2007, the peak season for HCoV-NL63 infection was in September-October, which was earlier than the peak for HCoV-OC43 infections (December-January). HCoV-NL63-infected patients were younger and more likely to have croup, febrile convulsions, and acute gastroenteritis. The majority of local HCoV-NL63 isolates were phylogenetically closely related to those found in Belgium and The Netherlands. In conclusion, HCoV-NL63 is an important yet uncommon virus among our hospitalized children with acute RTIs.

Acute respiratory tract infections (RTIs) are responsible for considerable morbidity and mortality in humans. A few families of viruses, namely, the *Paramyxoviridae*, *Orthomyxoviridae*, *Picornaviridae*, *Adenoviridae*, and *Coronaviridae* families, account for most of the infections (19). However, despite the use of a wide range of sensitive assays, the etiological agents of a large proportion RTIs still cannot be identified. It is not uncommon to detect cytopathic effects in viral cell cultures but negative results for known viruses (24). Even in the most comprehensive studies, a causative agent (either viral or nonviral) could be identified in only 85% of the patients (14). In part, this might be due to the limitations of diagnostic assays, but a proportion of RTIs might be caused by still unknown pathogens. For instance, our recent study found that about 45% of nasopharyngeal aspirates (NPAs) collected from children hospitalized for RTIs were virus negative (5).

Coronaviruses (CoVs), members of the genus *Coronavirus* of the *Coronaviridae* family, are enveloped viruses with a large plus-strand RNA genome. Three serologically distinct groups of CoVs have been described, and viruses within each group are classified by their host range and genome sequence. Up to 2003, three human CoVs (HCoVs) were known: HCoV-229E and HCoV-OC43 were identified in the mid-1960s and cause the common cold (11, 17, 23). The third HCoV is the recently identified severe acute respiratory syndrome CoV that caused life-threatening pneumonia with significant morbidity and mortality worldwide (4, 15).

Fouchier et al. identified a novel CoV from an 8-month-old boy with pneumonia in 2004. Unlike HCoV-229E and HCoV-OC43, that virus replicated efficiently in tertiary monkey kidney cells and Vero cells. The genome sequence of this CoV was most closely related to the genome sequences of porcine epidemic diarrhea virus and HCoV-229E. This novel virus, subsequently named HCoV-NL63 (GenBank accession number AY518894), could be detected in 3% of 139 patients with acute RTIs of unknown etiology (10). In another study, van der Hoek et al. described HCoV-NL63 in a 7-month-old child with bronchiolitis and conjunctivitis. Five of their eight HCoV-NL63-infected cases were younger than 1 year old (26). All of their positive isolates were found during the winter of 2002, giving rise to a detection frequency of 7%. On the other hand, none of their 306 samples collected in the spring and summer of 2003 contained HCoV-NL63. Subsequently, several groups reported that HCoV-NL63 was detected in 2 to 9% of stored respiratory specimens from patients suffering from a wide spectrum of acute RTIs (2, 3, 6–8, 18). The detection rate for HCoV-NL63 was higher among infants and young children (3, 6, 8). In published studies, coinfection with HCoV-NL63 and other respiratory viruses was found in up to 25% of cases (2, 3). From those studies, HCoV-NL63 was found to be associated with acute RTIs ranging from the common cold and bronchiolitis to pneumonia. However, the epidemiology, seasonality,

* Corresponding author. Mailing address: Department of Paediatrics, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, N.T., Hong Kong. Phone: 852-2632 2981. Fax: 852-2636 0020. E-mail: tfleung@cuhk.edu.hk.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 16 September 2009.

clinical course, and outcomes, as well as the laboratory features, of HCoV-NL63 infection were far from clear. Furthermore, all except one of the published studies were based on retrospectively collected data (2, 3, 6–8, 16, 18).

The objectives of this study were (i) to understand the seasonality and epidemiology of HCoV-NL63 in local children requiring hospitalization; (ii) to characterize the genetic diversity of HCoV-NL63 circulating in Hong Kong; and (iii) to delineate the demographic, clinical, and laboratory parameters that were associated with adverse outcomes in children with this emerging viral infection.

MATERIALS AND METHODS

Study populations. All recruited subjects were children younger than 18 years of age hospitalized with clinical features of acute RTI. This study was divided into two phases. During the first phase of retrospective case finding, 40 cases were randomly selected each week during a 12-month period in 2005–2006 for testing for HCoV infection. In the second phase of this study, 20 randomly selected cases were prospectively recruited each week during the subsequent 12-month period in 2006–2007. Our interim virologic testing at the end of the retrospective phase could not detect any HCoV-NL63 infections among 179 patients with wheezing illnesses and infections with respiratory viruses (influenza A and B viruses, respiratory syncytial virus [RSV], parainfluenza viruses types 1 to 3, or adenovirus) identified by immunofluorescence (IF). Thus, our prospective phase recruited only patients who were negative for these common respiratory viruses. The epidemiological, clinical, and laboratory features of these subjects were collected prospectively; and their NPA specimens were tested on the day of collection. Blinded to the HCoV-NL63 findings, the responsible pediatricians filled in the clinical data collection form. The requests for laboratory tests such as complete blood count and differential and C-reactive protein level were made when they were clinically indicated. These parameters were captured from the Hospital Authority Clinical Management System currently installed for use in our hospital. The patients' parents gave informed written consent, and the Clinical Research Ethics Committee of our university approved this study.

Collection and processing of NPA samples. The NPA samples from the subjects were sent to the laboratory in our in-house viral transport medium, which contains Hanks buffer, 0.5% bovine albumin, 0.1 mg/ml gentamicin, 500 IU/ml penicillin, 500 IU/ml streptomycin, 2.5 μg/ml amphotericin B (Fungizone), and $NaHCO_3$ (titrated to neutral pH) These samples were stored frozen at −80°C (for the retrospective study) or were analyzed fresh on the day of collection (for the prospective study).

Molecular detection for HCoVs. RNA was extracted from the NPA samples with a PureLink viral RNA/DNA minikit (Invitrogen, Carlsbad, CA). The extracted RNA was then transcribed into cDNA by using random hexamers with Superscript III RNase H-negative reverse transcriptase (Invitrogen), according to the manufacturer's instructions. In view of the high degree of genetic diversity of CoVs, this study adopted, with modifications, the laboratory protocols for a low-stringency pan-CoV reverse transcription-PCR (RT-PCR) assay targeting the polymerase gene for the detection of HCoVs in respiratory samples (18). This low-stringency RT-PCR identified viral sequences that were identical or closely related to the published HCoV-NL63 strain (10, 18, 26). Tables S1 and S2 in the supplemental material describe the mixture of 12 pairs of forward and reverse primers used for the RT-PCR assay. These primers were designed from the sequence alignment of 14 closely related CoVs. Table S1 in the supplemental material shows the primer-targeting gene positions on the sequence alignment of the 14 different strains of CoV starting from the 5′ position and going in the 3′ direction. After subtraction of the duplicate forward and reverse sequences from Table S1 in the supplemental material, the remaining 12 sequences were used to represent the sequences of the 14 different CoV strains for the design of primers within this region. Table S2 in the supplemental material shows the exact primer sequences derived from Table S1 in the supplemental material. The forward primers are in the 5′ to 3′ direction and the reverse primers are also reported in the 5′ to 3′ direction and are complementary to that of the primer positions shown in Table S1 in the supplemental material. PCRs were carried out in a total volume of 25 μl containing 2 μl cDNA, 0.25 μl HotStart *Taq*, 10 μM of each deoxynucleotide triphosphate, and 0.6 μl of each primer mixture. The samples were denatured at 94°C for 10 min, followed by 35 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 60 s and with a final extension at 72°C for 10 min. Positive and negative controls were included in each PCR experiment. The PCR products were electrophoresed in a 2% agarose gel. The expected size of the PCR products was 228 bp. This pan-CoV RT-PCR assay was used to screen for CoVs in NPA samples collected from both the retrospective and the prospective cohorts.

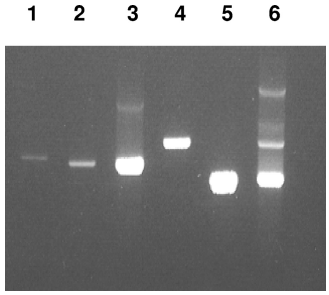


FIG. 1. Photograph of single and nested PCRs for 1a gene (lanes 4 to 6) and spike gene (lanes 1 to 3) of HCoV-NL63. Lane 1, PCR with outer primers only (698 bp); lane 2, PCR with inner primers only (663 bp); lane 3, nested PCR (663 bp); lane 4, PCR with outer primers only (839 bp); lane 5, PCR with inner primers only (525 bp); lane 6, nested PCR (525 bp).

Following this low-stringency RT-PCR assay, amplification products found to be positive for HCoVs were purified on a MicroSpin S400 HR column (Pharmacia Biotech, Sweden) and were subjected to direct gene sequencing with the forward primer mixture (see Table S2 in the supplemental material) and an ABI BigDye Terminator cycle sequencing kit (version 3) on an ABI 3130 sequencer (Applied Biosystems, Foster City, CA). The gene sequence obtained from each sample was compared with the publicly accessible sequences by using the nucleotide blast software of the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST) to confirm the identity.

Genetic diversity of HCoV-NL63 isolates. Samples positive for HCoV-NL63 were then reamplified for the partial 1a gene and partial spike gene regions by nested PCR. Table S3 in the supplemental material describes the primers used for these PCRs (18, 26). PCRs were carried out in a total volume of 25 μl containing 2 μl of the previously prepared cDNA, 0.25 μl HotStart *Taq*, 10 μM of each deoxynucleotide triphosphate, and 0.6 μl of each primer mixture. The samples were denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 60 s and with a final extension at 72°C for 10 min. The same PCR conditions were applied to both the 1a gene and the spike gene amplifications. Positive and negative controls were also included in each PCR experiment. The PCR products were then electrophoresed in 2% agarose gels. The expected PCR products were 525 bp (1a gene) and 663 bp (spike gene) (Fig. 1). The PCR products obtained were purified on a MicroSpin S400 HR column (Pharmacia Biotech) and were then subjected to sequencing on an ABI 3130 sequencer (Applied Biosystems) by using an ABI Prism BigDye Terminator cycle sequencing kit (version 3) (25).

Phylogenetic analysis. Sequence alignments of the partial 1a gene and partial spike gene sequences of HCoV-NL63 (10, 25) were generated by use of the CLUSTALW program (version 1.8) (22). Phylogenetic trees were constructed by the neighbor-joining method (21), and rooted phylogenetic trees were generated by using the PAUP* program (version 4.0 beta). The robustness of the phylogenetic trees was assessed by bootstrap analysis. Bootstrap values were determined with 1,000 resamplings of the data sets (9). Bootstrap values greater than 70% provide significant evidence of the phylogenetic grouping. The final tree was obtained with the FigTree program (version 1.2.2; http://tree.bio.ed.ac.uk/software/figtree/).

Statistical analysis. The results are expressed as means and standard deviations (SDs) or percentages, unless stated otherwise. Continuous variables between the different patient groups were compared by the Student *t* test, and categorical variables were analyzed by the chi-square or the Fisher exact test. Age, duration of hospitalization, and laboratory parameters between patients with and without HCoV-NL63 infection were compared by the nonparametric Mann-Whitney U test, owing to their likely skewed data distribution. The proportion of cases (those who were infected with HCoV-NL63) and controls (those who were NPA negative) and the clinical features of children infected with HCoV-NL63 compared with the clinical features of children not infected with any virus were compared by the chi-square test or the Fisher exact test. Multivariate logistic regression was used to analyze the patients' diagnoses that were independently associated with HCoV-NL63 infection, adjusted for age and sex as covariates. All analyses were done two tailed by using SPSS for Windows (version 14; SPSS Inc., Chicago, IL), with the level of significance set at 0.05.

TABLE 1. Clinical and laboratory features of subjects recruited in 2005–2006[a]

Feature	Result for the following children:		P
	NPA positive (n = 179)	NPA negative[b] (n = 1,802)	
Age (yr)	2.6 ± 2.8	3.5 ± 3.6	0.001
Male (%)	57.0	56.8	0.968
Duration of hospitalization (days)	3.5 ± 8.0	3.4 ± 5.4	0.835
Duration of fever (days)	3.1 ± 3.0	3.7 ± 3.4	0.044
Clinical status (% of subjects)			
Fever (temp ≥ 38°C) on admission	5.2	13.2	0.002
Shortness of breath	49.3	48.6	0.921
Cough	18.6	14.7	0.181
Minimum arterial O_2 saturation <95%	4.1	10.1	0.010
Abnormal chest radiograph	17.0	31.9	<0.001
Oxygen supplementation	0	5.3	<0.001
Laboratory results			
Hemoglobin concn (g/dl)	12.3 ± 1.2	12.4 ± 1.2	0.325
Platelet count (10^9/liter)	298.0 ± 154.6	307.5 ± 116.1	0.481
Total white cell count (10^9/liter)	9.1 ± 4.5	11.2 ± 6.1	<0.001
Absolute neutrophil count (10^9/liter)	4.88 ± 3.70	6.81 ± 5.31	<0.001
Absolute lymphocyte count (10^9/liter)	3.26 ± 2.51	3.19 ± 2.33	0.756
Highest C-reactive protein concn (mg/liter)	22.1 ± 31.8	36.3 ± 52.0	0.019

[a] The results are expressed as means ± SDs, unless stated otherwise.
[b] Cases negative for all viruses tested, including influenza A and B viruses, RSV, parainfluenza virus types 1 to 3, and adenovirus.

Nucleotide sequence accession numbers. The sequence data obtained in this study have been deposited in the GenBank database under accession nos. GQ856815 to GQ856831 (1a gene) and GQ856798 to GQ856814 (spike gene).

RESULTS

Study populations. Altogether, 1,981 NPA samples collected in 2005–2006 were studied. These included 179 NPA samples that had tested positive for seven common respiratory viruses by IF, as determined by tests performed as part of our routine investigations for RTIs. Table 1 summarizes the subjects' clinical and laboratory features. The mean (SD) ages of the NPA-positive and NPA-negative cases were 2.6 (2.8) years and 3.5 (3.6) years, respectively (P = 0.001). NPA-negative patients were more likely to have a fever (P = 0.002), a low minimum arterial O_2 saturation (P = 0.010), an abnormal findings on a chest radiograph (P < 0.001), and oxygen supplementation (P < 0.001). The two groups were comparable for temperature, blood pressure, and pulse and respiratory rates; but the NPA-negative cases had a higher total white cell count (P < 0.001), absolute neutrophil count (P < 0.001), and peak serum C-reactive protein concentration (P = 0.019). NPA-positive and -negative patients were matched for the need for intensive care and mortality (P = 1.0 for both). One thousand one cases were recruited during the second prospective phase. All of them were negative for respiratory viruses by our routine IF detection method. None of these patients died or required intensive care.

Detection of HCoV-NL63. Altogether, HCoVs were detected in 74 of the 2,982 (2.5%) cases studied: 46 from the retrospective cohort and 28 from the prospective cohort (P = 0.792). Table 2 summarizes the different HCoVs detected in our subjects. The sequences of all isolates from our subjects showed ≥93% homology with known HCoV sequences. Five (0.3%) and 12 (1.2%) patients infected with HCoV-NL63 were identified in 2005–2006 and 2006–2007, respectively (P = 0.006). The positive detection rates for the other three HCoVs were comparable in those 2 years.

TABLE 2. Identification of HCoVs among subjects in 2005–2006 and 2006–2007[a]

HCoV serotype	No. (%) of subjects		P^b
	2005–2006 (n = 1,802)	2006–2007 (n = 1,001)	
HCoV-NL63	5 (0.3)	12 (1.2)	0.006
HCoV-OC43	25 (1.4)	12 (1.2)	0.805
HCoV-229E	6 (0.3)	0	0.095
HCoV-HKU1	10 (0.6)	4 (0.4)	0.781
Total	46 (2.6)	28 (2.8)	0.792

[a] Only data for cases that tested negative for common respiratory viruses, including influenza A and B viruses, RSV, parainfluenza viruses, and adenovirus, are shown.
[b] P values for all serotypes except HCoV-229E were determined by the chi-square test; for HCoV-229E, the results were compared by the Fisher exact test.

Seasonality and clinical features of HCoV-NL63 infection. Altogether, the combined retrospective and prospective phases covered a consecutive 24-month period from 2005 to 2007 and identified 74 HCoVs for seasonality and epidemiological analyses. Figure 2 summarizes the seasonality patterns of the different HCoVs in this population. The incidence of HCoV-OC43 infections peaked in the winters (November to January) of 2005 to 2007, whereas the incidence of HCoV-NL63 infections peaked earlier in autumn (September-October), during this 2-year period. HCoV-HKU1 and HCoV-229E occurred at a much lower incidence, and no obvious peak could be observed. May, June, and July comprised the low season for all HCoVs.

Due to possible recall bias, the small number (n = 5) of patients with HCoV-NL63 infection in our retrospective cohort were not counted in our analysis for the associations between this virus infection and various clinical and laboratory conditions. Table 3 summarizes the prospective data collected from HCoV-NL63-infected cases and cases negative for all viruses recruited in 2006–2007. Patients with HCoV-NL63 infection were younger (P = 0.007) and were more likely to have a discharge diagnosis of croup (P < 0.001), febrile convulsion (P = 0.027), or acute gastroenteritis (P = 0.028). The presence of HCoV-NL63, however, did not show a significant association with chest radiographic abnormalities or any laboratory parameter (data not shown). On logistic regression analysis, HCoV-NL63 infection remained significantly associated with the three diagnoses listed above (P = 0.004, 0.036, and 0.042, respectively). As febrile convulsion occurred only in children younger than 6 years of age, we also repeated the analysis for the significant relationship with HCoV-NL63 infection in this age subgroup. There was still a trend toward such an association (20.0% versus 4.9%; P = 0.063). The association between HCoV-NL63 infection and croup remained significant (8.3% versus 0.4%; P < 0.001) among these younger children.

The possible associations between clinical and laboratory variables and infections by the other three HCoVs (n = 16) were also analyzed. Patients infected with other HCoVs had

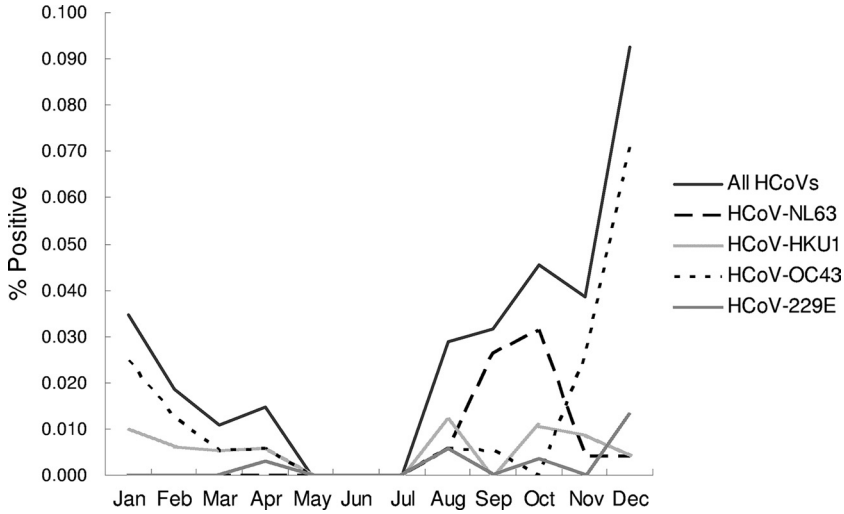


FIG. 2. Seasonality pattern for all HCoVs and individual serologic types in NPA samples collected during the 24-month study period from 2005 to 2007.

shorter durations of hospitalization than NPA-negative cases (median, 2.0 days [interquartile range, 0.3 to 2.8 days] versus 2.0 days [interquartile range, 1.0 to 4.0 days]; $P = 0.019$). The differences in all other clinical and laboratory parameters, however, were insignificant between these two groups ($P > 0.1$).

A total of 179 patients with acute bronchiolitis, bronchitis, or exacerbations of asthma in our retrospective cohort who had tested positive for respiratory viruses by IF were subjected to testing for HCoV to delineate the likelihood of any coinfection with common respiratory viruses. However, none of these patients were positive for HCoV-NL63. This detection rate was significantly lower than that for the NPA-negative subjects ($P = 0.019$ by Fisher exact test). This finding led us to focus only on subjects who had tested negative for the common respiratory viruses in the prospective phase of this study.

TABLE 3. Clinical and laboratory features of HCoV-NL63-infected and NPA-negative cases recruited during the prospective 12-month study period in 2006–2007[a]

Feature	Result for the following subjects:		P^b
	HCoV-NL63 infected ($n = 12$)	NPA negative[c] ($n = 989$)	
Age (yr)	0.9 (0.6–1.0)	2.0 (0.9–5.0)	0.007
Male (%)	41.7	46.3	0.751
Duration of hospitalization (days)	2.0 (1.0–2.8)	2.0 (1.0–4.0)	0.320
Duration of fever (days)	2.0 (1.0–3.0)	2.0 (1.0–4.0)	0.258
Fever on admission (% of subjects)	100	90.0	0.317
Discharge diagnoses (% of subjects)			
Upper respiratory tract infection	33.3	34.0	0.960
Acute tonsillitis	0	2.1	0.614
Croup	8.3	0.3	<0.001
Acute bronchiolitis	8.3	5.8	0.711
Acute bronchitis	0	0.6	0.784
Asthma exacerbations	8.3	6.5	0.803
Pneumonia	0	14.3	0.157
Acute otitis media	0	0.8	0.751
Febrile convulsion	16.7	3.9	0.027
Acute gastroenteritis	33.3	12.2	0.028
Roseola infantum	0	2.8	0.557
Viral illness	8.3	4.7	0.552

[a] The results are expressed as medians (interquartile ranges), unless stated otherwise.

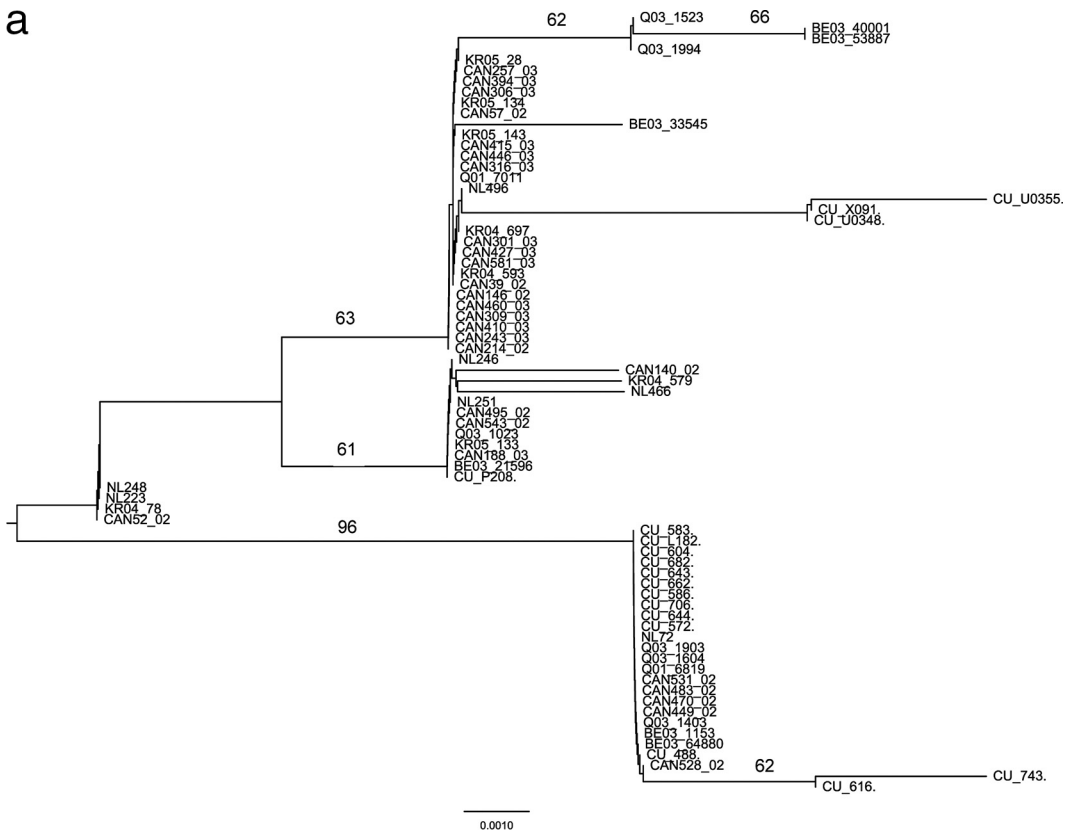
[b] Analyzed by the Mann-Whitney U test for numerical variables and the chi-square or Fisher exact test for categorical data.

[c] Cases negative for all viruses tested, including HCoV-NL63, influenza A and B viruses, RSV, parainfluenza viruses, and adenovirus.

Phylogenetics of HCoV-NL63 isolates. Figure 3a and b shows the phylogenetic trees for the partial 1a and partial spike gene sequences, respectively. The phylogenetic analysis of the 1a gene sequences suggested that the majority of HCoV-NL63 isolates circulating in Hong Kong were closely related to strains that have been reported in Belgium (strains BE-03-1153 and BE-03-64880) and The Netherlands (strain NL72). This observation was also revealed when the spike gene sequences were used to construct the phylogenetic tree. A small group of HCoV-NL63 strains (strains CU_U0348, CU_X091, CU_P208, and CU_U0355) circulating in Hong Kong was separated from the major cluster.

DISCUSSION

In 2004, two groups of researchers independently reported on the identification and characterization of HCoV-NL63 (10, 26). This novel virus was detected in 3% and 7% of their subjects, respectively. Subsequently, several groups found HCoV-NL63 in 2 to 8.8% of stored respiratory specimens from patients with acute RTIs (2, 3, 6–8, 18). In contrast, two recent studies suggested that this virus was less common (0.4%) than what we thought (16, 20). Among the published studies, coinfection with HCoV-NL63 and other respiratory viruses was found in up to 25% of cases (2, 3). Nonetheless, none of our 179 cases in the retrospective cohort positive for common respiratory viruses had HCoV-NL63 coinfection. One possible reason is that we have selected from the retrospective cohort only those cases who had wheezing disorders to test for HCoV-NL63, in view of the possible associations between this virus and exacerbations of asthma (12%) and acute bronchiolitis (6%), as reported in a local study (16). We might have missed HCoV-NL63 coinfections that occurred in children with other diagnoses.



There are four major serologically distinct groups of HCoVs: HCoV-OC43, HCoV-NL63, HCoV-229E, and HCoV-HKU1. A number of published studies showed that all of these viruses are capable of causing a variety of human diseases (6, 10, 11, 17, 23, 26). We addressed this issue by developing a low-stringency pan-CoV RT-PCR assay for the detection of HCoV-NL63 in NPA samples collected from local children. Recent reports on pan-CoV RT-PCRs targeting the polymerase and replicase 1a genes of CoVs helped us to develop the protocol (8, 18). This low-stringency RT-PCR allowed the identification of NPA samples containing viral sequences that were identical or closely related to those of all the known HCoVs (10, 26). The low stringency was accomplished by the use of a combination of nucleotide base degeneracy, a low concentration of magnesium, and a low annealing temperature. Our pan-CoV RT-PCR allowed us to screen for the presence of known HCoVs in an efficient way. Although the severe acute respiratory syndrome CoV is nonexistent in our population at present, this pan-CoV RT-PCR would also be able to identify that virus. On the basis of the conserved CoV sequences from HCoVs that infect different species, this pan-CoV system offers the advantage that it may be able to identify previously unknown CoVs. Although we did not test for the sensitivity of this RT-PCR assay, Moës and colleagues reported that it was capable of detecting 5×10^3 RNA copies per μl of NPA sample (18).

There were two published studies of HCoV-NL63 in Hong Kong. Chiu et al. reported the first local study of HCoV-NL63 in 2002–2003 that involved 587 children hospitalized for acute RTIs (6). HCoVs were detected in 26 (4.4%) of the children; and the rates of detection of HCoV-NL63, HCoV-OC43, and HCoV-229E were 2.6%, 1.5%, and 0.3%, respectively. HCoV-NL63 could present as croup, exacerbation of asthma, febrile seizures, and high fever. In that study, HCoV-NL63 infections were noted in the spring and summer months of 2002, whereas HCoV-OC43 infections mainly occurred in the fall and winter months of 2001. Lau et al. conducted a 12-month prospective study in 2004–2005 of 4,181 adults and children hospitalized for acute RTIs in two local regional hospitals (16). In contrast to the methods used in our study, they performed RT-PCRs with primers specific for HCoV-HKU1, HCoV-NL63, HCoV-OC43, and HCoV-229E. They found HCoVs in 87 (2.1%) patients, with 0.3% HCoV-HKU1, 0.4% HCoV-NL63, 1.3% HCoV-OC43, and 0.1% HCoV-229E infections being detected. Febrile seizures were more common in those infected with HCoV-HKU1 (50%) than in those infected with HCoV-OC43 (14%). The incidence of both viruses peaked in the winter of 2004–2005, whereas HCoV-NL63 infections occurred mainly in early summer and autumn. In our 24-month study conducted from 2005 to 2007, 74 (2.5%) patients were found to have HCoV infections. This incidence is comparable to that of the second local study described above (16). The two studies had similar rates of detection of individual HCoVs and also found a seasonality pattern of an autumn peak in HCoV-NL63 infections (Fig. 2). On the other hand, these findings were inconsistent with those reported in another local study (6) and in studies with Caucasians (18, 26). Selection bias is the likely reason that accounted for the high rate of detection of HCoV-NL63 in the latter studies (18, 26). Despite this, we found that HCoV-NL63 is associated with croup, febrile convulsions, and acute gastroenteritis in local children. As these diagnoses were not associated with HCoVs other than HCoV-NL63, our findings also suggest that the three disease associations described above are specific to HCoV-NL63 but not the other three HCoVs. Among these diagnoses, HCoV-NL63 also remained associated with croup when the data were adjusted by the use of the Bonferroni correction for multiple statistical comparisons. In contrast to the other HCoVs, there was a significant difference in the rates of detection of HCoV-NL63 during the two 12-month periods (Table 2). This finding is the beneficial result of our two-stage study design. The many published studies that have looked for HCoV-NL63 only in any 1-year period might produce inaccurate results concerning its epidemiology (6–8, 20).

CoV, being an RNA virus, has a high degree of genetic diversity. The current literature on the sequence variability of HCoV-NL63 isolates in Asian countries is limited. Phylogenetic analyses of the 1a gene sequences revealed that the majority of circulating HCoV-NL63 isolates are closely related to strains reported in The Netherlands (strain NL72) and Belgium (strains BE03_1153 and BE03_64880) (Fig. 3a). Whereas the former strain was known from a local study (6), the strains from Belgium were new in Hong Kong. Besides, a small proportion of HCoV-NL63 strains (strains CU_P208, CU_X091, CU_U0348, CU_U0355, CU_616, and CU_743) found in Hong Kong were phylogenetically separated from the major group. For the spike gene sequences, most local isolates were closely related to either the prototype strain found in The Netherlands (strain NL496) or the strains listed above, as was found by 1a gene sequence analysis (Fig. 3b). The possible implications of these findings on the design of PCR primers and other detection assays deserve further study. We suggest that representative samples from both clusters of isolates be included when diagnostic assays are evaluated.

The major limitation of this study is that a number of emerging respiratory viruses, such as metapneumovirus, bocavirus, and rhinovirus (1, 4, 12, 13), were not detected. Apart from HCoVs, this study attempted to identify only common respiratory viruses by IF, and no attempt was made to identify the three viruses listed above. We do not know the proportion of HCoV-infected patients who were also infected with those viruses. Therefore, we cannot confidently attribute our disease

FIG. 3. Phylogenetic tree of the HCoV-NL63 partial 1a gene (a) and spike gene (b) sequences. Bootstrap values, indicated at the nodes, were obtained from 1,000 bootstrap replicates and are reported as percentages. The scale bar corresponds to 0.001 substitution per site. The accession numbers for the reference strains are NC_005831, AY567488 to AY567494, AY675541 to AY675553, AY746451 to AY746458, AY758283 to AY758287, AY758297 to AY758301, DQ093116 to DQ093123, abd DQ106888 to DQ106901. CAN, Canada; Q, Australia; NL, The Netherlands; BE, Belgium; KR, South Korea. NL496 is the prototype sequence of HCoV-NL63. All sequences obtained from this study are named with a "CU" prefix. The GenBank accession numbers for our sequences are GQ856815 to GQ856831 for the 1a gene GQ856798 to GQ856814 for the spike gene.

associations with HCoV-NL63 to that virus alone or whether the diseases were caused by coinfection with HCoV-NL63 and other emerging viruses. In addition, the detection of a relatively small number of HCoV-NL63-infected cases in this study did not allow us to draw any definite conclusion on the clinical course of HCoV-NL63 infection and its association with clinical diseases.

In conclusion, HCoV-NL63 is uncommon among our hospitalized children with acute RTIs, being detected in 0.3% and 1.2% of these children in 2005–2006 and 2006–2007, respectively. The overall rate of detection for HCoVs during this period was 2.5%. HCoV-NL63 infections showed a peak in autumns (September-October) of 2005–2007, whereas infections with HCoV-OC43, the commonest HCoV, were found in the winter (November to January). Compared with acute RTI cases in which no virus was detected, patients with HCoV-NL63 infections are younger and are more likely to be diagnosed with croup, febrile convulsions, and acute gastroenteritis. This study suggests that the majority of the HCoV-NL63 isolates circulating in Hong Kong are phylogenetically closely related to the prototype strain found in The Netherlands (strain NL496). Nevertheless, a small proportion of the local HCoV-NL63 strains were phylogenetically distinct from the major cluster.

ACKNOWLEDGMENT

This study was supported by the Research Fund for the Control of Infectious Diseases (project no. 04050372), Food and Health Bureau of the Hong Kong SAR.

REFERENCES

1. **Allander, T., M. T. Tammi, M. Eriksson, A. Bjerkner, A. Tiveljung-Lindell, and B. Andersson.** 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc. Natl. Acad. Sci. USA **102:**12891–12896.
2. **Arden, K. E., M. D. Nissen, T. P. Sloots, and I. M. Mackay.** 2005. New human coronavirus, HCoV-NL63, associated with severe lower respiratory tract disease in Australia. J. Med. Virol. **75:**455–462.
3. **Bastien, N., K. Anderson, L. Hart, P. van Caeseele, K. Brandt, D. Milley, T. Hatchette, E. C. Weiss, and Y. Li.** 2005. Human coronavirus NL63 infection in Canada. J. Infect. Dis. **191:**503–506.
4. **Chan, P. K. S., J. S. Tam, C. W. Lam, E. Chan, A. Wu, C. K. Li, T. A. Buckley, K. C. Ng, G. M. Joynt, F. W. Cheng, K. F. To, N. Lee, D. S. Hui, J. L. Cheung, I. Chu, E. Liu, S. S. Chung, and J. J. Sung.** 2003. Human metapneumovirus detection in patients with severe acute respiratory syndrome. Emerg. Infect. Dis. **9:**1058–1063.
5. **Cheng, F. W., T. F. Leung, and J. S. Tam.** 2004. Comparison between disease spectrum of respiratory syncytial virus and human metapneumovirus infections in hospitalized children in Hong Kong, p. 27. Hong Kong Int. Conf. Infect. Dis. Food and Health Bureau, Hong Kong SAR.
6. **Chiu, S. S., K. H. Chan, K. W. Chu, S. W. Kwan, Y. Guan, L. L. Poon, and J. S. Peiris.** 2005. Human coronavirus NL63 infection and other coronavirus infections in children hospitalized with acute respiratory disease in Hong Kong, China. Clin. Infect. Dis. **40:**1721–1729.
7. **Ebihara, T., R. Endo, X. Ma, N. Ishiguro, and H. Kikuta.** 2005. Detection of human coronavirus NL63 in young children with bronchiolitis. J. Med. Virol. **75:**463–465.
8. **Esper, F., C. Weibel, D. Ferguson, M. L. Landry, and J. S. Kahn.** 2005. Evidence of a novel human coronavirus that is associated with respiratory tract disease in infants and young children. J. Infect. Dis. **191:**492–498.
9. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach utilizing the bootstrap. Evolution **39:**783–791.
10. **Fouchier, R. A., N. G. Hartwig, T. M. Bestebroer, B. Niemeyer, J. C. de Jong, J. H. Simon, and A. D. Osterhaus.** 2004. A previously undescribed coronavirus associated with respiratory disease in humans. Proc. Natl. Acad. Sci. USA **101:**6212–6216.
11. **Hendley, J. O., H. B. Fishburne, and J. M. Gwaltney, Jr.** 1972. Coronavirus infections in working adults. Eight-year study with 229 E and OC 43. Am. Rev. Respir. Dis. **105:**805–811.
12. **Ip, M., E. A. Nelson, E. S. Cheuk, E. Leung, R. Y. Sung, and P. K. Chan.** 2008. Pediatric hospitalization of acute respiratory tract infections with human bocavirus in Hong Kong. J. Clin. Virol. **42:**72–74.
13. **Jackson, D. J., R. E. Gangnon, M. D. Evans, K. A. Roberg, E. L. Anderson, T. E. Pappas, M. C. Printz, W. M. Lee, P. A. Shult, E. Reisdorf, K. T. Carlson-Dakes, L. P. Salazar, D. F. DaSilva, C. J. Tisler, J. E. Gern, and R. F. Lemanske, Jr.** 2008. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am. J. Respir. Crit. Care Med. **178:**667–672.
14. **Juven, T., J. Mertsola, M. Waris, M. Leinonen, O. Meurman, M. Roivainen, J. Eskola, P. Saikku, and O. Ruuskanen.** 2000. Etiology of community-acquired pneumonia in 254 hospitalized children. Pediatr. Infect. Dis. J. **19:**293–298.
15. **Kuiken, T., R. A. Fouchier, M. Schutten, G. F. Rimmelzwaan, G. van Amerongen, D. van Riel, J. D. Laman, T. de Jong, G. van Doornum, W. Lim, A. E. Ling, P. K. Chan, J. S. Tam, M. C. Zambon, R. Gopal, C. Drosten, S. van der Werf, N. Escriou, J. C. Manuguerra, K. Stöhr, J. S. Peiris, and A. D. Osterhaus.** 2003. Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. Lancet **362:**263–270.
16. **Lau, S. K., P. C. Woo, C. C. Yip, H. Tse, H. W. Tsoi, V. C. Cheng, P. Lee, B. S. Tang, C. H. Cheung, R. A. Lee, L. Y. So, Y. L. Lau, K. H. Chan, and K. Y. Yuen.** 2006. Coronavirus HKU1 and other coronavirus infections in Hong Kong. J. Clin. Microbiol. **44:**2063–2071.
17. **McIntosh, K., J. H. Dees, W. B. Becker, A. Z. Kapikian, and R. M. Chanock.** 1967. Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. Proc. Natl. Acad. Sci. USA **57:**933–940.
18. **Moës, E., L. Vijgen, E. Keyaerts, K. Zlateva, S. Li, P. Maes, K. Pyrc, B. Berkhout, L. van der Hoek, and M. Van Ranst.** 2005. A novel pancoronavirus RT-PCR assay: frequent detection of human coronavirus NL63 in children hospitalized with respiratory tract infections in Belgium. BMC Infect. Dis. **5:**6.
19. **Monto, A. S., and K. M. Sullivan.** 1993. Acute respiratory illness in the community. Frequency of illness and the agents involved. Epidemiol. Infect. **110:**145–160.
20. **Papa, A., E. Papadimitriou, L. K. Luna, M. Al Masri, E. Souliou, M. Eboriadou, A. Antoniadis, and C. Drosten.** 2007. Coronaviruses in children, Greece. Emerg. Infect. Dis. **13:**947–949.
21. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
22. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
23. **Tyrrell, D. A., and M. L. Bynoe.** 1965. Cultivation of novel type of common-cold virus in organ cultures. BMJ **i:**1467–1470.
24. **van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, R. A. Fouchier, and A. D. Osterhaus.** 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat. Med. **7:**719–724.
25. **van der Hoek, L., K. Pyrc, and B. Berkhout.** 2006. Human coronavirus NL63, a new respiratory virus. FEMS Microbiol. Rev. **30:**760–773.
26. **van der Hoek, L., K. Pyrc, M. F. Jebbink, W. Vermeulen-Oost, R. J. Berkhout, K. C. Wolthers, P. M. Wertheim-van Dillen, J. Kaandorp, J. Spaargaren, and B. Berkhout.** 2004. Identification of a new human coronavirus. Nat. Med. **10:**368–373.