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Human coronavirus NL63 in children: epidemiology, disease spectrum, and genetic diversity

Key Messages

1. Human coronaviruses (HCoV) were detected in 2.5% of 2982 local children hospitalised for acute respiratory infections in 2005 to 2007.
2. Using the 'pancoronavirus' reverse transcription-polymerase chain reaction assay, detection rates were 0.6% for HCoV-NL63, 1.2% for HCoV-OC43, 0.5% for HCoV-HKU1, and 0.2% for HCoV-229E. Notably, HCoV-NL63 infections were significantly more common among children hospitalised in 2006/2007 (1.2%) than in 2005/2006 (0.3%).
3. The peak season for HCoV-NL63 infection was autumn (September to October).
4. HCoV-NL63 infection was associated with younger age, croup, febrile convulsion, and acute gastroenteritis. Such disease associations were not found with the other three HCoVs.
5. Most local HCoV-NL63 isolates were closely related to the prototype strain in Netherlands (NL496), but a few were phylogenetically distinct from the major cluster.

Introduction

Acute respiratory tract infections (RTIs) account for considerable morbidity and mortality in humans. Even in the most comprehensive studies, a causative agent (either viral or non-viral) can be identified in only 85% of the patients. This may be due to the limitations of diagnostic assays, but a proportion of RTIs may be caused by unknown pathogens. Coronaviruses, a genus of the *Coronaviridae* family, are enveloped viruses with a large plus-strand RNA genome. Up to the year 2003, three serologically distinct groups of human coronaviruses (HCoVs) had been described: HCoV-229E, HCoV-OC43, and SARS-CoV. Two independent groups identified HCoV-NL63 in the following year.^{1,2} Among Caucasians, this virus could be detected in 2 to 8.8% of patients with acute RTIs of 'unknown aetiology'. Most positive isolates were found in winter, and this virus might be associated with a wide range of acute RTIs (from common cold to pneumonia). Nonetheless, the epidemiology, seasonality, and clinical features of HCoV-NL63 infection remain unclear.

We aimed to (1) understand the seasonality and epidemiology of HCoV-NL63 in local children, (2) characterise the genetic diversity of HCoV-NL63 circulating in Hong Kong, and (3) delineate demographic, clinical, and laboratory parameters of such infections in our population.

Methods

This two-phased study was conducted from 1 February 2006 to 1 July 2008. In the first phase, about 2000 systematically selected nasopharyngeal aspirate (NPA) samples from hospitalised children (<18 years old) with acute RTIs during a 12-month period in 2005/2006 were retrospectively screened for all types of HCoVs. In the second phase, NPA samples from the first 20 hospitalised children in every week with un-identified common respiratory viruses were prospectively collected between December 2006 and November 2007. Clinical and demographic data of 1001 subjects with virus-negative NPA samples were obtained. Laboratory tests were requested when clinically indicated. No therapeutic intervention was carried out. Patients' parents gave informed written consent, and the Joint CUHK-NTEC Clinical Research Ethics Committee approved this study.

Identification of HCoVs in nasopharyngeal aspirate samples

The NPA samples were stored at -80°C or analysed fresh. Extracted RNA was transcribed into cDNA using random hexamers with Superscript III RNase H-reverse transcriptase (Invitrogen, Carlsbad [CA], USA). For the detection of HCoVs, a low-stringency 'pancoronavirus' RT-PCR assay targeted against the polymerase gene was developed.³ A mixture of 12 pairs of forward and reverse primers were designed from the sequence alignment of 15 closely related CoVs. PCR reactions 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 mix. Samples were denatured at 94°C for 10 minutes followed by 35 cycles of 94°C for 40 seconds, 55°C for 40 seconds and 72°C for 60 seconds, and a final extension at 72°C for 10 minutes. The 228-bp PCR products were electrophoresed in 2% agarose gels. Following this, amplification products

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positive for HCoV were purified using MicroSpin S400 HR column (Pharmacia, Biotech, Sweden) and subjected to gene sequencing using the forward primer mix and BigDye Terminator Cycle sequencing kit v.3 on an ABI-3130 Sequencer (Applied Biosystems, Foster City [CA], USA). The gene sequence thus obtained from each subject was compared with publicly accessible sequences, using nucleotide blast software of Blastn Program (<http://www.ncbi.nlm.nih.gov/BLAST>) to identify the HCoV with the highest percentage of sequence homology.

Genetic diversity of HCoV-NL63 isolates

HCoV-NL63-positive samples were re-amplified for the partial 1a and partial spike genes using nested-PCR.³ The same PCR reaction mixes as above were used. Samples were denatured at 94°C for 5 minutes followed by 35 cycles

of 94°C for 40 seconds, 50°C for 40 seconds, and 72°C for 60 seconds, and a final extension at 72°C for 10 minutes. The expected PCR products were 525 bp (1a gene) and 663 bp (spike gene), respectively. The PCR products obtained were purified using a MicroSpin S400 HR column, and then subjected to direct sequencing on an ABI-3130 Sequencer.

Phylogenetic analysis

Sequence alignments of partial 1a and spike gene sequences of HCoV-NL63¹ were generated by CLUSTALW (version 1.8). Phylogenetic trees were constructed by the neighbour-joining method, rooted phylogenetic trees were generated using PAUP* (version 4.0; beta). The robustness of phylogenetic trees was assessed by bootstrap analysis. Bootstrap values were determined with 1000 resamplings of the data sets. Bootstrap values greater than 70% provide

Table 1. Identification of HCoVs among nasopharyngeal aspirate-negative subjects in 2005/2006 and 2006/2007

HCoV serotype	No. (%) of positive subjects		P value*
	2005/2006 (n=1802)	2006/2007 (n=1001)	
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

* Chi squared test for all except for HCoV-229E, which used the Fisher exact test

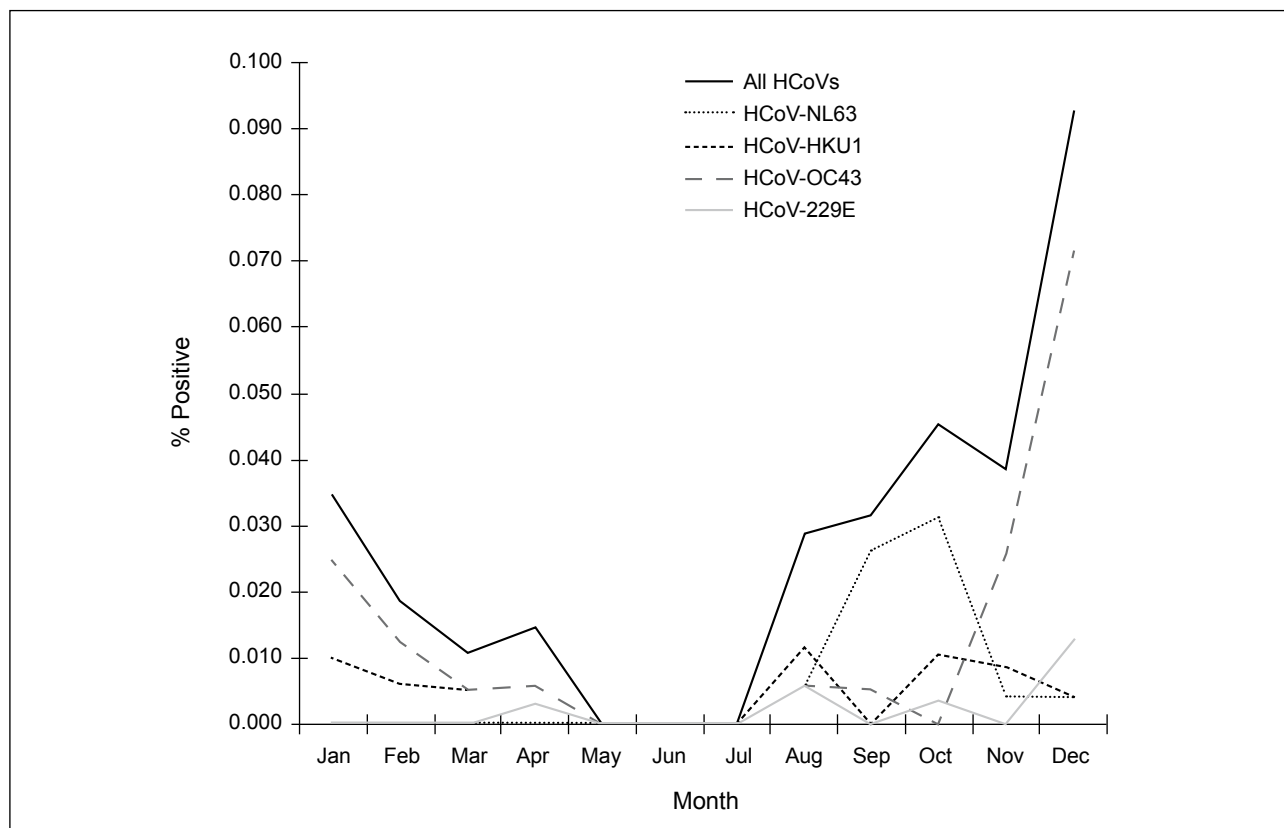


Fig. Seasonality pattern for all HCoVs and individual serologic types in nasopharyngeal aspirate samples collected during the 24-month period in 2005 to 2007

Table 2. HCoV-NL63-infected and nasopharyngeal aspirate (NPA)-negative cases recruited during the 12-month period in 2006/2007

Feature	HCoV-NL63-infected*	NPA-negative*	P value†
Patient age (years)	0.9 (0.6-1.0)	2.0 (0.9-5.0)	0.007
Male	41.7	46.3	0.751
Duration of hospitalisation (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	100	90.0	0.317
Abnormal chest radiograph	25.0	38.3	0.441
Discharge diagnoses			
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
Laboratory results			
Haemoglobin (g/dL)	12.3 (11.3-13.6)	12.3 (11.4-13.0)	0.726
Platelet count (x10 ⁹ /L)	347.0 (285.0-431.8)	293.0 (231.0-374.3)	0.110
Total white cell count (x10 ⁹ /L)	9.5 (8.4-11.4)	9.5 (6.6-13.4)	0.864
Absolute neutrophil count (x10 ⁹ /L)	4.49 (2.55-6.77)	5.39 (3.10-8.93)	0.300
Absolute lymphocyte count (x10 ⁹ /L)	2.82 (2.21-4.55)	2.53 (1.58-4.10)	0.234
Highest C-reactive protein (mg/L)	7.7 (1.4-82.9)	23.2 (7.3-66.1)	0.137

* Data are presented as median (interquartile range) or %

† Analysed by Mann-Whitney *U* test for numerical variables, and Chi squared or Fisher exact test for categorical data

significant evidence for phylogenetic grouping. The final tree was obtained with the FigTree program (version 1.2.2) (<http://tree.bio.ed.ac.uk/software/figtree/>).

Virus isolation for HCoV-NL63

Although there is currently no established method for isolating HCoV-NL63, we attempted to propagate five HCoV-NL63 in a few commonly used cell lines including Vero, MDCK, LLC-MK2, HeLa, Hep2, RD, and MRC-5 cells. Briefly, 0.3 mL of NPA was inoculated onto each cell monolayer and incubated at 37°C with maintenance medium containing 1 µg/mL of trypsin. Cell monolayers were examined daily for 14 days for cytopathic effect. The medium changed on days 5 to 7. All specimens were blind passaged once.

Results

Of 1981 NPA samples, 1802 were NPA negative and 179 were patients with acute bronchiolitis, bronchitis or asthma exacerbations whose NPA had ≥1 respiratory virus by direct immunofluorescence. The mean±standard deviation age of NPA-positive and NPA-negative subjects were 2.6±2.8 and 3.5±3.6 years, respectively ($P=0.001$). NPA-negative patients were more likely to have fever ($P=0.002$), low SaO₂ ($P=0.010$), abnormal chest radiograph ($P<0.001$), and oxygen supplementation ($P<0.001$). The two groups were comparable for their vital signs, but NPA-negative cases had higher total white cell counts ($P<0.001$), absolute neutrophil counts ($P<0.001$), and peak serum C-reactive protein levels ($P=0.019$).

Detection of HCoV-NL63

Our 'pancoronavirus' RT-PCR identified 74 (2.5%) cases as having HCoVs among all 2982 NPA samples. Of these HCoV-infected patients, 46 and 28 came from the retrospective and prospective cohorts, respectively ($P=0.792$). A total of 17 (0.6%) patients with HCoV-NL63 were identified in 2005 to 2007, being significantly more common among children hospitalised in 2006/2007 than in 2005/2006 (1.2% vs 0.3%, $P=0.006$, Table 1). The detection rates for the other three types of HCoVs were comparable in these 2 years. In our retrospective cohort, none of the 179 NPA-positive patients with acute bronchiolitis, bronchitis or asthma exacerbations had co-infection with HCoV-NL63.

Seasonality and clinical features of HCoV-NL63 infection

A total of 74 HCoVs were identified in the combined 24-month study period from 2005 to 2007. The incidence of HCoV infections among our hospitalised children peaked in winter (November to January), which was mainly associated with HCoV-OC43 infection (Fig). In contrast, the peak season for HCoV-NL63 infection in local children occurred earlier in autumn (September to October) during the same period.

In view of any possible recall bias, five patients with HCoV-NL63 infection in our retrospective cohort were not counted. Patients with HCoV-NL63 infection were younger ($P=0.007$), and more likely to have had croup ($P<0.001$), febrile convulsion ($P=0.027$) and acute gastroenteritis ($P=0.028$) [Table 2]. Such disease associations were not

detected with the other three HCoV (P>0.1). Nonetheless, HCoV-NL63 was not associated with any significant laboratory abnormality.

Phylogenetics of HCoV-NL63 isolates

The majority of HCoV-NL63 isolates circulating in Hong Kong in 2005 to 2007 were closely related phylogenetically to strains that have been reported in Belgium (BE-03-1153, BE-03-64880) and the Netherlands (NL72). A small group of HCoV-NL63 strains (CU U0348, CU X091, CU P208, CU U0355) that circulated in Hong Kong were distinct from the major cluster.

Virus isolation for HCoV-NL63

Despite blind passage, none of the cell lines used showed evidence of virus growth either by cytopathic effect or RT-PCR determination at the end of incubation.

Discussion

Two groups of researchers independently identified HCoV-NL63 in 2004.^{1,2} This virus was present in 2 to 8.8% of respiratory specimens from patients with acute RTIs. Another study suggested that this virus was less common (0.4%).⁴ There are four major serologically distinct groups of HCoVs: HCoV-OC43, HCoV-NL63, HCoV-229E, and HCoV-HKU1. In this study, we developed a low-stringency RT-PCR assay for the simultaneous detection of these HCoVs. This 'pancoronavirus' system may also be able to identify previously unknown CoVs.

In 587 hospitalised children, 26 (4.4%) HCoVs were identified, and 2.6% were HCoV-NL63.⁵ The peak season for infection was in spring and summer of 2002. In 2004/2005, HCoVs were found in 2.1% of adults and children hospitalised for acute RTIs, with HCoV-NL63 accounting for 0.4%.⁴ Both HCoV-HKU1 and HCoV-OC43 infections peaked in winter, whereas HCoV-NL63 infections occurred mainly in early summer and autumn. In the present study, 74 (2.5%) patients were detected to have HCoV infection in 2005 to 2007. The seasonality pattern for HCoV-NL63

was consistent with another study (Fig).⁴ Interestingly, the detection rates of HCoV-NL63 were significantly different in the two 12-month periods (Table 1). Thus, studies that look for HCoV-NL63 in any 1-year period only might produce inaccurate epidemiological results.⁵

CoV, being an RNA virus, has a high degree of genetic diversity. Phylogenetic analyses of 1a and spike gene sequences revealed that most circulating HCoV-NL63 were closely related to strains in Netherlands (NL72 and NL496) and Belgium (BE03 1153, BE03 64880).⁵ A small proportion of the strains circulating in Hong Kong were phylogenetically distinct from the major group.^{1,2} We suggest including representative samples from both clusters of isolates when evaluating diagnostic assays.

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