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The dominance of human coronavirus OC43 and NL63 infections in infants

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ABSTRACT

Background: It is unknown to what extent the human coronaviruses (HCoVs) OC43, HKU1, 229E and NL63 infect healthy children. Frequencies of infections are only known for hospitalized children.
Objectives: Comparing infection frequencies in children who have mild infections with frequencies in children needing hospital uptake will determine whether infection by one of the four HCoVs leads to more severe disease. In addition, the sequence of seroconversions can reveal whether infection by one HCoV protects from infection by other HCoVs.
Study design: Two distinct study groups were monitored: healthy children and children hospitalized due to respiratory infection. HCoV natural infection rates in healthy children were obtained by serology in 25 newborns (followed 0–20 months). The frequencies of severe HCoVs infection was determined by real time RT-PCR among 1471 hospitalized infants (<2-years old) with acute respiratory tract disease.
Results: The majority of healthy children seroconverted for HCoV-OC43 ($n = 19$) and HCoV-NL63 ($n = 17$), less for HCoV-HKU1 ($n = 9$) and HCoV-229E ($n = 5$). Notably, HCoV-HKU1 seroconversion was absent after HCoV-OC43 infection. Also HCoV-229E infection was rarely observed after HCoV-NL63 infection (1 out of 5). In the hospital 207 (14%) out of 1471 children were HCoV positive. Again we observed most infection by HCoV-OC43 ($n = 85$) and HCoV-NL63 ($n = 60$), followed by HCoV-HKU1 ($n = 47$) and HCoV-229E ($n = 15$).
Conclusions: HCoV-NL63 and HCoV-OC43 infections occur frequently in early childhood, more often than HCoV-HKU1 or HCoV-229E infections. HCoV-OC43 and HCoV-NL63 may elicit immunity that protects from subsequent HCoV-HKU1 and HCoV-229E infection, respectively, which would explain why HCoV-OC43 and HCoV-NL63 are the most frequently infecting HCoVs. There are no indications that infection by one of the HCoVs is more pathogenic than others.

1. Background

Human coronaviruses (HCoV) NL63, 229E, OC43 and HKU1 are circulating worldwide among the human population and cause approximately 10% of all upper and lower respiratory tract illnesses.[1–3] In children, infections with HCoV-NL63, HCoV-229E, HCoV-OC43 and HCoV-HKU1 are associated with acute respiratory tract illness, pneumonia and croup that eventually may lead to hospitalization.[4]

The severe acute respiratory syndrome (SARS) outbreak in 2002/2003 by a novel coronavirus, followed by the recent identification of HCoV-NL63 (2004) and HCoV-HKU1 (2005) renewed research interest into HCoV infections and their ability to seriously affect human health.[5–8] Despite the accumulating knowledge on HCoV prevalence and burden of disease, there are limited studies on the frequency of infection by all 4 HCoV infections in the non-hospitalized population during the first years of childhood.[9–11]

2. Objectives

We established a specific carboxyl-terminal nucleocapsid (NCt) protein ELISA system for HCoV-OC43 and HCoV-HKU1 analogous to that described for HCoV-NL63 and HCoV-229E.[12] With this serological toolset we performed a survey with longitudinal sera from newborns to identify seroconversion events during the first years of

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life. We compared the serology data with the frequencies of infection of all 4 HCoVs in hospitalized infants with acute respiratory tract disease. In addition the chain of seroconversions will reveal whether immunity to one HCoV may protect against infection by one of the other HCoVs.

3. Study design

3.1. Patient samples

Two distinct study groups were monitored: healthy children (newborns) and children hospitalized due to respiratory disease. Human serum specimens from newborns were collected at the department of Medical Microbiology, Academic Medical Center (AMC), Laboratory of Experimental Virology. All children (12 males and 13 females) were born to HIV-1-positive mothers, with various dates of birth (1993, $n=1$; 1997, $n=1$; 1998, $n=3$; 1999, $n=4$; 2000, $n=1$; 2001, $n=1$; 2002, $n=4$; 2003, $n=7$; and 2004, $n=3$). In a previous study we compared the average age of seroconversion in the children born from HIV infected mothers and those born from healthy mothers. The mean seroconversion age was not different,[10] therefore we treated this group of 25 children as a representative of the wider population. Serum samples were obtained at birth, age 1 month, 3 months, 12 months, approximately 20 months, and for some also at approximately 24 months. Serum samples were stored at −80 °C. All newborns remained HIV-1 RNA negative and were HIV-1 seronegative during the follow-up period. Twenty-four of the 25 children were never hospitalized during the follow up period. One child was hospitalized in the first month of life due to an influenza infection. So none of the 25 children needed hospitalization at the moment they were infected by the HCoVs. Thirteen of the 25 newborns were part of a previous survey on HCoV-NL63 and HCoV-229E seroconversion.[10] Respiratory samples were not collected. All serum samples were heat-inactivated at 56 °C for 30 min.

Respiratory samples from children hospitalized due to respiratory infection, were collected and analyzed at the Elisabeth Hospital, Tilburg, Netherlands ($n=168$) and The Edinburgh Royal Infirmary (South-east of Scotland) ($n=1303$) for routine respiratory virus screening.[13–15] Samples had been collected during 5 consecutive years. Samples in this study were selected from the complete set based on the following criteria: children who were hospitalized with acute respiratory tract illness and below the age of 2 years. This provides a selection of children of which the HCoV infections are severe enough to require hospitalization and who encountered their primary HCoV infection.

3.2. Generation and expression of recombinant HCoV carboxyl-terminal nucleocapsid proteins

In this study the carboxyl-terminal region of the nucleocapsid (NCt) protein was used as antigen, instead of the previous used full length N protein.[10] This was done to increase the specificity of the serological assay. In a previous study we show that the carboxyl part of the N protein of 229E and NL63 elicit specific antibodies that allow differentiation between both species.[16] Given the shared structural and functional domain features among coronavirus N proteins we applied the same approach for HCoV-OC43 and HCoV-HKU1, since cross-reactivity by the antibodies directed to the full-N of HCoV-OC43 and HCoV-HKU1 has been reported in human sera.[16–18] The HCoV-NL63 and HCoV-229E recombinant NCt proteins were produced as previously described.[12] The generation of the plasmid for NCt-OC43 and NCt-HKU1 was performed using the same method. Briefly, for NCt-OC43 the following primer combination was used 5′ OC43_N5_CT (5′-CACCAGATTAGAGTTGGCCAAAGTG-3′) and 3′ OC43_Nexp (5′-TTATATTTCTGAGGTGTCTTCAGTATAG-3′), whereas for NCt-HKU1 the primer combination 5′-HKU1_5N_CT (5′-CACCAAATTAGACTTGGTTAAAAGAGTCCG-3′) and 3′ HKU1_Nexp (5′-TTAAGCAACAGAGTCTTCTACATAAG-3′) was used. The generated pET100_OC43_NCt and pET100_HKU1_NCt plasmids were sequenced and shown to be 100% identical to the virus reference sequences of HCoV-HKU1 (Caen1, HM034837) and HCoV-OC43 (VR-759, AY391777), respectively. Subsequent expression and purification of the HCoV recombinant carboxyl-terminal N (NCt) proteins were performed exactly as described elsewhere.[10] The NCt proteins from HCoV-OC43, HCoV-HKU1, like those from HCoV-NL63 and HCoV-229E, retained their immunogenicity in ELISA (checked with longitudinal sera from adults, or pooled human intravenous immunoglobulin (IVIg, Sanquin)). We performed competition experiments to ascertain specificity: positive human serum from adults or human IVIG was diluted (1:200) in PBST containing 1% skim milk, and twofold serial dilutions (ranging from 0 to 50 μg/ml) of NCt protein of HCoV-NL63, HCoV-229E, HCoV-OC43, or HCoV-HKU1 were added. The mixtures were briefly homogenized by vortexing prior to incubation for 1 h at RT. Following the preincubations, the samples were measured by either NL63- and 229E-ELISA or OC43- and HKU1-ELISA (so preincubated with the NCt form the other virus of the same genus). No cross reactivity was observed.

3.3. Carboxyl-terminal nucleocapsid ELISA

The procedure for the NCt ELISA of HCoV-NL63, HCoV-OC43, HCoV-HKU1 and HCoV-229E was performed as previously described.[10,12] Briefly, 96-well ELISA plates (Greiner Bio-one) were coated overnight at 4 °C with 3 μg/ml of expressed recombinant NCt protein. Non-specific binding sites were blocked with phosphate-buffered saline-0.1% Tween 20 (PBST) supplemented with 5% skim milk (Fluka) for 1 h at room temperature (RT). Longitudinal serum samples were diluted 1:200 in PBST containing 1% skim milk and incubated in the plate for 2 h at RT. After a washing, alkaline phosphatase-conjugated anti-human immunoglobulin G Fcγ-tail antibody (Jackson Immunoresearch) diluted (1:1500) in 1% skim milk–PBST was added. Following 1 h at RT, the plates were washed and signal was developed with 50 μl of Lumi-Phos Plus (Lumigen). Measurements were done with a Glomax 96 plate luminometer (Promega). All sera were tested in duplicate. Seroconversion was defined as a signal increase of >2.5 compared to that of the preceding time point.

3.4. Statistical analysis

Calculations were performed using the Prism software version 5 (Graphpad). Comparison of longitudinal results from the cumulative incidence of HCoV-OC43, HCoV-HKU1, HCoV-NL63 and HCoV-229E seropositive time points was done with the Kaplan–Meier survival analysis, statistical significance was tested with the log-rank (Mantel–Cox) test. The time point of seroconversion was calculated by taking the midpoint between the last seronegative and the first seropositive time point. Comparison of the mean prevalence for HCoV-OC43, HCoV-HKU1, HCoV-NL63 and HCoV-229E infections among children under the age of 2 years was done with one-way ANOVA, using the Turkey's multiple comparison test.

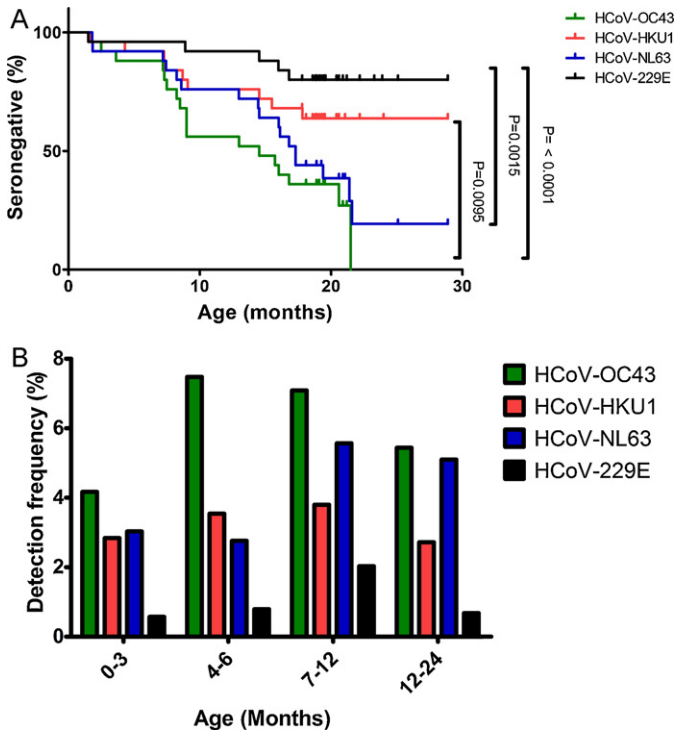

Fig. 1. Frequency of infection by the four HCoVs in children. (A) Healthy children: seroconversion for HCoV-OC43, HCoV-NL63, HCoV-HKU1, HCoV-229E during the complete follow up period. The Kaplan–Meier survival analysis was performed on the cumulative incidence of the percentage of seronegative individuals (*y*-axis) against time (in months; *x*-axis). Seroconversion to HCoV-NL63 is presented as a blue line, HCoV-OC43 as a green line, HCoV-229E as a black line and HCoV-HKU1 as a red line. (B) Hospitalized children: percentage of coronavirus HCoV-229E, HCoV-HKU1, HCoV-NL63, and HCoV-OC43 positive patients by age group as determined by viral RNA diagnostics. HCoV-NL63, HCoV-OC43, HCoV-229E and HCoV-HKU1 are presented as a blue, green, black and red bar, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

4. Results

4.1. Seroconversion during childhood

In this serological survey we measured the antibody levels towards all four HCoVs in sera collected from 25 newborns who were followed until an average of 20 months of age. During this period none of the children were hospitalized due to an HCoV infection. At birth we observed high levels of antibodies directed to all four HCoVs that decreased to low detectable levels within a few months. This suggests that all newborns carry maternal antibodies directed to all four HCoVs. In one newborn (Subject 1) we did not observe any seroconversion for the HCoVs during 19 months follow up (Table 1). For 5 newborns a single seroconversion was detected to one of the four HCoVs, whereas for the remaining newborns we observed sequential or simultaneous seroconversion events. In total, we recorded 17 events of seroconversion towards HCoV-NL63, 19 for HCoV-OC43, 5 for HCoV-229E and 9 towards HCoV-HKU1 (Table 1). No correlation was found between the antibody levels of some of the mothers before and after giving birth and the seroconversion outcome of their children (data not shown).

To determine whether there is variability in seroconversion rates for the different HCoV infections we used the cumulative incidence of seroconversion for each virus (Fig. 1A), using the mid-time point of each event (Table 1). Statistical analyses (Log-rank, Mantel Cox test) revealed that there are significant differences in the frequencies of the four HCoVs. HCoV-OC43 seroconversions occurred significantly more frequently than HCoV-HKU1 ($p = 0.0095$) and HCoV-229E infections ($p \leq 0.0001$), but not HCoV-NL63 infections ($p = 0.1773$). For HCoV-NL63 the frequency of seroconversion was significantly higher compared with HCoV-229E ($p = 0.0015$). No significant difference in seroconversion rates between alpha- (NL63 and 229E) or betacoronavirus (OC43 and HKU1) was found ($p = 0.1529$).

4.2. Infection frequencies versus disease severity

Our antibody data showed that seroconversion to HCoV-OC43 and HCoV-NL63 occurs most frequently. These seroconversion frequencies can be compared with the frequencies with which these viruses are found in hospitalized children – children of the same age group that appear with respiratory symptoms so severe that hospitalization was needed frequently. This study covered 5 consecutive years, and children were included at two locations: The Netherlands and South-east of Scotland. Between December 2006 and February 2011 a total of 207 (14%) out of 1471 children were HCoV infected. HCoV-OC43 ($n = 85$) was most frequently detected, followed by HCoV-NL63 ($n = 60$), HCoV-HKU1 ($n = 47$) and HCoV-229E ($n = 15$) (Fig. 1B). The frequency of HCoV-229E infection was significantly lower compared to HCoV-NL63 ($p \leq 0.05$) and HCoV-OC43 ($p \leq 0.001$). The frequency of HCoV-HKU1 infection was significantly lower compared to HCoV-OC43 ($p \leq 0.05$). Thus, in hospitalized children under the age of 2 years, HCoV-OC43 and HCoV-NL63 were the most common coronaviruses detected, similar to the higher rates of seroconversion events observed for these two viruses.

4.3. Sequence of seroconversions

HCoV-NL63 and HCoV-229E belong to the alphacoronaviruses, whereas HCoV-OC43 and HCoV-HKU1 are members of the betacoronaviruses. These groups were originally designed on serological reactivity, suggesting that antibodies could cross-react with the other virus from the same group (we were aware of potential cross reactivity and carefully designed the partial NCt protein ELISA to best specificity, see Section 3). Antibodies directed to the Spike protein have the potential to be neutralizing, and in case these antibodies cross-react, seroconversion towards one HCoV might protect against infection by the other virus from the same group.

Inspection of the NL63/229E seroconversion dates shows that child 7 seroconverted for HCoV-NL63 6 months after HCoV-229E (December 2004 versus June 2004), suggesting that an HCoV-229E infection did not protect from HCoV-NL63 infection. Further inspection showed that in none of the HCoV-229E seroconversions a recent infection by HCoV-NL63 was noted (≤1 year before HCoV-229E infection) also pointing towards HCoV-NL63 eliciting protective immunity for HCoV-229E infection. Strikingly, exactly the same pattern is observed for OC43/HKU1. HCoV-HKU1 seroconversions are only observed prior to HCoV-OC43 seroconversion, telling that also for these two viruses an infection by HCoV-OC43 elicits immunity that is protective towards an HCoV-HKU1 infection. This is not vice versa, child 6, 7, 10, and 21 seroconvert for HCoV-OC43 while they had been infected previously by HCoV-HKU1, suggesting that an infection with HCoV-HKU1 does not protect against an HCoV-OC43 infection.

5. Discussion

This is the first study on the seroconversion frequencies of all four HCoVs. In healthy children high frequency of seroconversion towards HCoV-OC43 and HCoV-NL63 were detected, and to a lesser extent seroconversion to HCoV-HKU1 and HCoV-229E. Serosurveillance of otherwise healthy individuals subverts bias for analyses of

Table 1
Mid-time points of seroconversion.

Subject	Follow-up period			HCoV-NL63		HCoV-229E		HCoV-OC43		HCoV-HKU1	
	Start	End	Duration (months)	Age[a]	Date[b]	Age[a]	Date[b]	Age[a]	Date[b]	Age[a]	Date[b]
1	Sept-03	Mar-05	18.9								
2	Oct-02	Jul-04	20.9							15.5	Jan-04
3	Apr-04	Mar-06	24.0	21.4	Jan-06			7.3	Nov-04		
4	Jul-03	Mar-05	20.6					1.8	Sept-03		
5	Jul-02	Dec-04	28.9					13.0	Aug-03		
6	Jan-04	Feb-06	25.1					9.0	Oct-04	4.3	May-04
7	Feb-03	Aug-05	21.6	21.6	Dec-04	16.0	Jun-04	21.6	Dec-04	8.7	Oct-03
8	Aug-03	May-05	21.2	17.3	Jan-05					7.4	Mar-04
9	Oct-02	Apr-04	18.6	17.3	Mar-04			9.0	Jul-03		
10	Jun-02	Jun-04	23.9	19.4	Jan-04			8.5	Feb-03	1.6	Aug-02
11	Oct-03	May-05	18.8	16.8	Mar-05			9.0	Jul-04		
12	Aug-03	Mar-05	18.1			8.9	May-04				
13	Sept-03	May-05	19.4	8.6	Jan-04	16.8	Feb-05	16.8	Feb-05		
14	Jul-99	Feb-01	19.4	13.0	Aug-00					17.8	Dec-00
15	Nov-00	Jul-02	19.3					2.5	Feb-01		
16	Jul-93	Feb-95	19.5	7.4	Feb-94			15.7	Oct-95		
17	Dec-97	Jul-99	18.8	14.4	Feb-99			2.6	Mar-98		
18	Aug-98	Feb-00	17.8	7.2	Feb-99			7.2	Feb-99		
19	Aug-98	Jun-00	22.2	16.1	Dec-99			20.6	May-00		
20	Jun-04	Jan-06	19.1	8.2	Feb-05			8.2	Feb-05		
21	Aug-99	Apr-01	19.6	16.0	Jan01			16.0	Jan-01	7.3	Apr-00
22	Aug-98	Jul-00	22.4	14.5	Oct-99	14.5	Oct-99	14.5	Oct-99	14.5	Oct-99
23	Dec-99	Sept-01	21.1			1.51	Feb-00	7.5	Aug-00		
24	Dec-99	Sept-01	20.4	1.8	Feb-00			15.7	May-01		
25	Jan-01	Aug-02	19.1	1.8	Mar-01					9.1	Oct-01

[a] Age of seroconversion in months.
[b] Date of seroconversion.

patients with higher severity of symptoms as is an inevitable consequence of hospital based studies, thus providing a much clearer representation of virus epidemiology in the community. These data provide the opportunity to compare the natural infection frequency of each HCoV with the frequency with which these viruses are found in hospitalized children with acute respiratory infections within a community, something that has not been achieved previously. We investigated children who were hospitalized with acute respiratory tract illness, below the age of 2 years. This provides a selection of children with severe HCoV infections requiring hospitalization. Prevalence data in these hospital children and the serology in the healthy children are in complete accordance, revealing that there are no indications that any of the four HCoVs causes significantly more hospitalization than another.

The characteristic frequency of infection, in the order HCoV-OC43 ≥ HCoV-NL63 > HCoV-HKU1 ≥ HCoV-229E, observed via seroconversion but also by direct detection of the virus in hospitalized children over multiple years is in contrast with some previous studies. Some studies addressed the frequency of HCoV infection during childhood and suggested that HCoV-NL63 infections were associated with more hospitalization compared to HCoV-OC43.[3,19,20] In the majority of the abovementioned studies only 1 year was sampled and for HCoVs a large year-to-year periodicity is known.[14,21,22] The significant differences of seroconversion rates between the coronaviruses observed here cannot be attributed to variable circulating frequencies as samples were collected over multiple years.

There has never been a clear explanation for the higher frequency of infection for HCoV-OC43 and HCoV-NL63 compared to HCoV-HKU1 and HCoV-229E among children. We hypothesize that an infection by HCoV-NL63 elicits neutralizing antibodies directed to the NL63-Spike protein that might also protect, or partially protect, against an HCoV-229E infection, whereas this relationship may not be reciprocated, thus providing a greater likelihood of HCoV-NL63 infection than HCoV-229E. The same can count for HCoV-OC43 for which neutralizing antibodies may protect against HCoV-HKU1 infection. The seroconversion data we show here support our hypothesis, yet confirmation with in vitro neutralization studies is needed.

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Competing interests

None declared.

Ethical approval

Not required.

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