Human coronavirus NL63 and 229E seroconversion in children

Ronald Dijkman¹, Maarten F. Jebbink¹, Nawal Bahia El Idrissi¹, Krzysztof Pyrc², Marcel A. Müller³, Taco W. Kuijpers⁴, Hans L. Zaaijer⁵ and Lia van der Hoek*¹

¹Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, The Netherlands
²Microbiology Department, Faculty of Biochemistry Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
³Institute for Virology, University of Bonn Medical Centre, Bonn, Germany
⁴Department of Pediatric Hematology, Immunology and Infectious Disease, Emma Children’s Hospital, Academic Medical Center, Amsterdam, The Netherlands
⁵Laboratory of Clinical Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, The Netherlands

*Correspondence and requests for material should be addressed to Lia van der Hoek, Laboratory of Experimental Virology, University of Amsterdam, Meibergdreef 15, 1105 AZ, Amsterdam, The Netherlands; Phone number: +31 20 566 75 10; Fax: +31 20 691 65 31; c.m.vanderhoek@amc.uva.nl
Abstract
In 2004, a novel respiratory human coronavirus (HCoV) NL63 was identified and subsequent research revealed that the virus has spread worldwide, as its close relative HCoV-229E. HCoV-NL63 and HCoV-229E infections can lead to the hospitalization of young children, immunocompromised persons and elderly. Children infected with HCoV-NL63 often develop croup, with obstruction of the airway. In this study we investigated at which age children are confronted for the first time with an HCoV-NL63 infection and, thus, at which age they seroconvert to HCoV-NL63. We designed a recombinant HCoV-229E and a recombinant HCoV-NL63 nucleocapsid protein ELISA and performed a seroepidemiology survey on longitudinal and cross-sectional sera. The longitudinal sera were collected from 13 newborns, from which multiple time points were available spanning a period of at least 18 months. For the cross-sectional survey we tested sera of 139 children between the age of 0 to 16 years. In the longitudinal serum samples we observed that all children have maternal anti-NL63 and anti-229E antibodies at birth that disappear within 3 months. Seven of the thirteen children become HCoV-NL63 seropositive during follow up, whereas only 2 become HCoV-229E seropositive. The serology data of the cross-sectional serum samples revealed that 75% and 65% of the children in the age group 2.5 – 3.5 years are HCoV-NL63 and HCoV-229E seropositive, respectively. Concluding, HCoV-NL63 and HCoV-229E seroconversion occurs on average before children reach the age of 3.5 years.
Introduction

Coronaviruses (CoVs) are enveloped, plus-strand RNA viruses belonging to the family Coronaviridae (12). The genomic RNA is 27 – 32 Kb in size, capped and polyadenylated. The virions have a unique morphology, with extended, petal-shaped spikes that give the virus a crown-like projection (Latin; corona) under the electron microscope (12). All coronaviruses (CoVs) possess a common genome organization where the replicate gene encompasses the 5’-two thirds of the genome and is comprised of two overlapping open reading frames (ORFs), ORF1a and ORF1b. The structural gene region, which covers the 3’-third of the genome, encodes the canonical set of structural protein genes in the order 5’ - spike (S) - envelope (E) - membrane (M) and nucleocapsid (N) – 3’. The S and N proteins are the most abundantly expressed during virus infections and both induce an immune response (12). CoVs are classified into three groups based on phylogenetic and serological relationships (12). Group 1 and 2 consist of various mammalian coronaviruses, whereas avian viruses cluster in group 3.

Nowadays, there are five different human coronaviruses (HCoVs) known, all of which are associated with respiratory tract infections in humans. HCoV-229E (a group 1 coronavirus) and HCoV-OC43 (group 2) were discovered in 1966 and 1967, respectively (9, 14). Both HCoVs were identified as the causative agents of the common cold in humans by experimentally inoculation of healthy adult volunteers (1-3, 11, 17). Almost 40 years later the Severe Acute Respiratory Syndrome (SARS) outbreak emerged. The causative agent was identified as a novel member of the group 2 CoVs (5). Over 8000 SARS-CoV infections were reported during the peak period of the 2002 and 2003 outbreak with a mortality rate of 10% (5). After revealing that highly pathogenic HCoVs can evolve, efforts of identification and characterization of new HCoVs increased. This resulted in the identification of two new family members in 2004 and 2005, HCoV-NL63 (group 1) and HCoV-HKU1 (group 2), respectively (21, 24). Infections with either HCoV-HKU1 or HCoV-NL63 can lead to hospitalization of young children, elderly and immunocompromised patients (21). In addition, HCoV-NL63 is associated with croup, which is a common manifestation of lower respiratory tract infections with a peak occurrence in the first two life years (22). Globally, approximately 10% of all upper and lower respiratory tract infections in hospitalized children is caused by HCoV-229E, HCoV-OC43, HCoV-HKU1 and HCoV-NL63 infections (8, 20).

Clinical studies indicated that HCoV-NL63 infections in children frequently occur below the age of 3 years (22). These studies encompassed children that were hospitalized due to respiratory tract infections. This sketches a situation that HCoV-NL63 infections in children will lead to hospitalization, however, we hypothesise that HCoV-NL63 infections are common during childhood and that only a minor fraction of infections requires hospitalization of children. Retrospective, investigation of all HCoV-NL63 infections in children is possible with a serological assay, like ELISA. The age and frequency of infection can be monitored by measurement of HCoV-NL63 antibody titer rise (seroconversion). The nucleocapsid (N) protein of CoVs is rather conserved within species, it is immunogenic, and it induces one of the strongest immune response of all structural proteins (25). The HCoV-NL63 N protein shares only 42% amino acid identity with the N protein of its closest relative HCoV-229E (16), therefore an N protein ELISA is likely to be species specific.
We developed an HCoV-NL63 N protein ELISA and determined that this assay is species specific. We observed no cross-reactivity between HCoV-NL63 N directed antibodies and HCoV-229E N directed antibodies, thus confirmed the specificity of the ELISA. Subsequently, we performed seroepidemiological surveys with longitudinal and cross-sectional sera obtained from newborns and children between 0 – 16 years old, respectively, to determine at which age children seroconvert to HCoV-NL63 and thus are confronted for the first time with an HCoV-NL63 infection.
Material & Methods

Serum for longitudinal and cross-sectional surveys
Human serum specimens from newborns were collected at the department of Medical Microbiology, Academical Medical Center (AMC), Laboratory of Experimental Virology. All children were born to HIV-1-positive mothers. Serial samples were collected during a follow-up period of at least 18 months and stored at -80°C. All 13 children remained HIV-1 RNA negative and became HIV-1 seronegative during the follow-up period.

Human serum specimens from individuals between the ages of 0 – 16 years old were collected at the department of Medical Microbiology, AMC, Laboratory of Clinical Virology. Sera were obtained between 1999 and 2003 and were stored at -80°C. All serum samples were heat-inactivated at 56°C for 30 minutes.

Preparation of recombinant HCoV-NL63 and HCoV-229E nucleocapsid protein expression constructs
HCoV-NL63 RNA was isolated and reverse transcribed as described (15). The cDNA was used as template for generation of full length 1134 nt N-gene with the primer combination 5’ NL63_Nexp (5’ – CACCGCTAGTGTAATAATGGGC – 3’) and 3’ NL63_Nexp (5’ – TTAATGCACACCTCGTTGAC – 3’). The pTRE-HN plasmid (kind gift from Volker Thiel, (18)) was used as template for amplification of the full length 1170 nt N gene of HCoV-229E with the following primer combination 5’ 229E_Nexp (5’ – CACCGCTACAGTCAAATGGGCT – 3’) and 3’ 229E_Nexp (5’ – TTAGTTTACTTCATCAATTAT – 3’). Amplification of the N genes was performed with Pfx polymerase (Invitrogen). Amplified N gene fragments were cloned in the pET-100D-TOPO vector (Invitrogen). The generated pET100_NL63 and pET100_229N plasmids were sequenced and shown to be 100% identical to the virus reference sequences of HCoV-NL63 (Amsterdam-01; NC_005831) and HCoV-229E (Inf-1; NC_002645), respectively.

Expression of HCoV-NL63 and 229E nucleocapsid proteins
Expression of recombinant nucleocapsid proteins of HCoV-NL63 and HCoV-229E was performed by transformation of 10 ng plasmid in chemically prepared competent E. Coli BL-21 derived strain Rosetta™ 2 (Novagen). Vector coding for the recombinant LacZ protein (using pET100/D/LacZ plasmid, Invitrogen) was included as a control. Overnight cultures of transformed bacteria containing either pET100_229N, pET100_NL63N or pET100_LacZ plasmid were inoculated into Luria Broth (LB) medium, supplemented with 1% glucose, carbenicillin (10 µg /ml) and chloramphenicol (17,5 µg/ml). Cultures were grown to exponential phase, prior to induction with 0.5 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 hours. Recombinant proteins were purified with Ni-NTA Agarose (Qiagen), and protein concentrations were determined with the Micro BCA protein assay (Pierce).
Nucleocapsid ELISA
Ninety-six-well ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 3 µg/ml of expressed recombinant N protein of HCoV-NL63 or HCoV-229E or LacZ protein (negative control). The proteins were diluted in 0,1 M carbonate buffer pH 9.6. Unspecific binding sites were blocked with PBS + 0,1% Tween20 (PBST) supplemented with 5% skim milk (Fluka) for one hour at room temperature (RT). Longitudinal and cross-sectional sera were diluted 1:200 or 1:100, respectively, in PBST containing 1% skim milk and incubated on the plate for 2 hours at RT. After washing, Alkaline Phosphatase conjugated anti-human IgG Fcy-tail antibody (Jackson immunoresearch) diluted (1:1500) in 1% skim milk PBST was added. Following one hour 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. In the study with cross sectional sera a cutoff value was used. This value was the mean from the levels of the 6 – 12 months old children in either HCoV-NL63 or HCoV-229E ELISA.

Nucleocapsid competition ELISA
Human sera were diluted (1:200) in PBST containing 1% skim milk and twofold serial dilutions (ranging from 50 to 0 µg/ml) of either expressed recombinant N protein of HCoV-NL63, N protein of HCoV-229E or LacZ protein were added. The mixtures were briefly homogenized by vortexing prior to incubation for 1 hour at RT. No centrifugation was performed. Following the pre-incubations the samples were measured by HCoV-NL63 or HCoV-229E ELISA, as described above.

Statistical analysis
Calculations were performed using the Prism software version 5 (Graphpad). The median inhibition concentration (IC_{50}) of the soluble HCoV-NL63 N, HCoV-229E N and LacZ protein competitor in the competition ELISA was calculated with the non-linear regression method, with variable slope. Comparison of longitudinal results from the cumulative incidence of 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. Comparison of the HCoV-NL63 and HCoV-229E analysis with cross-sectional sera was performed with the non-parametric Mann-Witney U test to determine if there was statistical difference between the frequency of seroconversion to HCoV-NL63 and HCoV-229E.
Results

Comparison of the N protein sequences of HCoV-NL63 (YP_003771) and its closest relative HCoV-229E (NP_073556) revealed that these proteins share only 42% similarity. We hypothesized that the difference on amino acid level is sufficiently high for a recombinant HCoV-NL63 N protein ELISA specific for HCoV-NL63 N protein directed antibodies. To verify the specificity, we performed a competition ELISA with coated HCoV-NL63 N-protein and incubated several HCoV-NL63 antibody positive samples with serial dilutions of LacZ, HCoV-NL63 N or HCoV-229E N proteins. To exclude sample to sample variation we performed the competition ELISAs for 4 different serum samples. For all four samples we observed that incubation with the homologous protein (HCoV-NL63 N protein) did inhibit the HCoV-NL63 ELISA (IC_{50} ranging between 9 to 43 µg /ml), whereas the heterologous proteins did not (IC_{50} >50 µg /ml for both HCoV-229E N and LacZ protein). So, in the competition assay we observed that only HCoV-NL63 N protein pre-incubation diminished the signal in the HCoV-NL63 N ELISA. Incubation with HCoV-229E protein or LacZ had no effect in the HCoV-NL63 ELISA, while HCoV-229E pre-incubation did inhibit an HCoV-229E N ELISA (shown for serum 1 in Figure 1). Thus, no antigenic cross-reactivity between HCoV-NL63 N-antibodies and HCoV-229E N-antibodies has been observed.

We aimed to investigate the age of a first HCoV-NL63 infection and therefore we followed newborn children and determined the age at which antibody titers to HCoV-NL63 rise. We have access to sequential serum samples of newborn children that are followed until the age of 1.5 years (ranging from 18 months to 29 months). Serum samples were collected approximately at month 0, 1, 3, 12, and 18. The first thing that was noted was the high level of antibodies at birth. All children carried maternal HCoV-NL63 N antibodies. The level of these antibodies decreased to very low levels within a few months. Seroconversion to HCoV-NL63 during follow up was seen for 7 of the 13 children (Figure 2, panel C, G, H, I, J, K and M). To determine whether seroconversion to HCoV-229E occurs as frequent as seroconversion to HCoV-NL63, the same longitudinal set of samples was tested in an HCoV-229E specific N protein ELISA. Similar to the HCoV-NL63 analysis we observed that all children carry maternal HCoV-229E antibodies at birth and that the level of antibodies decreased within the first months of life. Only 2 of the 13 children seroconverted to HCoV-229E (Figure 2, panel G and L) during follow up. Interestingly, one child presented a sequential antibody level increase to both HCoV-229E and HCoV-NL63, at month 10 and 26 respectively (Figure 2, panel G). Five of the 13 children did not seroconvert to HCoV-NL63 or HCoV-229E during follow up. To investigate whether seroconversion to HCoV-NL63 occurs significantly earlier or more frequent than to HCoV-229E we determined the cumulative incidence (Kaplan-Meier) of seroconversion for each virus (Figure 3). The statistical analysis revealed however, that the difference of the frequency of infection by each virus is not significantly different (P=0.08).

To investigate the frequency of infection of both viruses at higher ages, we collected a total of 139 sera from children between the ages of 0 – 16 years old and performed a cross-sectional survey. We observed that the majority (64%) of infants younger than 6 months of age have N protein directed antibodies against both viruses (Figure 4). These N protein directed antibodies are most probably maternal antibodies. The numbers of seropositive individuals in the 6 – 12 months age category are
low for both HCoV-NL63 and HCoV-229E, 33% and 22% respectively. These percentages only slightly change in the age group of 1 – 1.5 years, the number of seropositive individuals for HCoV-229E increases to 36%, whereas for HCoV-NL63 it decreases to 29%. At the age 1.5 to 3.5 years most children become infected by HCoV-NL63 and HCoV-229E. The percentage of seropositive children increases almost 2 fold to 65% for HCoV-229E and 75% for HCoV-NL63. Beyond the age of 3.5 years we observed that the vast majority carries antibodies directed to HCoV-NL63 and HCoV-229E (Figure 4). Like the longitudinal survey, we did not observe a significant difference between the frequency of infection between HCoV-NL63 and HCoV-229E.
Discussion

Here we show that HCoV-NL63 infections are common during childhood, and the majority of seroconversion to HCoV-NL63 occurs before children reach the age of 3.5 years. This finding is comparable to what is known for other respiratory viruses like human metapneumovirus (hMPV), respiratory syncytial virus (RSV) and human bocavirus (HBoV). The approximate age of seroconversion for these viral pathogens varies and seems to peak between the age groups 5 – 10, 2 – 5 and 2 – 6 years for hMPV, RSV and HBoV, respectively (6, 7). We observed that the majority of children older than 10 years is seropositive for HCoV-NL63, which is similar as the observations for the previously mentioned viral pathogens (6, 7). In conclusion, HCoV-NL63 circulates among the entire children population, and, HCoV-NL63 plays an important role in acquired childhood infections.

The antibody specificity directed towards viral proteins determines which virus-specific antigen can be used in a serological assay. For HCoV-NL63, it has recently been published that human sera contain antibodies directed against full length recombinant expressed HCoV-NL63 N protein (23). Vlasova et al reported that HCoV-NL63 N protein directed antibodies displayed no antigenic cross-reactivity with the N protein of non-human CoVs (23). However, it was not determined whether HCoV-NL63 directed N protein antibodies could cross-react with full length recombinant expressed HCoV-229E N protein. The CoVs N protein directed antibodies do not have neutralizing potential due to the structural blueprint where the N proteins are located inside the enveloped virion (12), thus, antigenic cross-reactivity can only be determined by performing a competition ELISA and not with a neutralization assay. With the competition assay we observed no detectable antigenic cross-reactivity between HCoV-NL63 N-protein directed antibodies and HCoV-229E N protein directed antibodies. A similar finding has recently been published by another research group (13). Therefore the utilization of HCoV N proteins in an ELISA provides a convenient tool for analyzing the seroepidemiological profiles of HCoV-NL63 and HCoV-229E.

The analysis of longitudinal sera from children allowed us to simultaneously measure the HCoV-NL63 and HCoV-229E directed antibodies levels directly after birth with a follow-up period of at least 18 months. In all children we detected high levels of maternal HCoV-NL63 and HCoV-229E N protein directed antibodies at birth, although, the degree of maternal antibody titers varied between newborns. Nonetheless, these antibody levels decreased to low levels within a period of 3 months and remained low until subsequent infection. Five of the thirteen children remained HCoV-NL63 and HCoV-229E seronegative during follow-up. We investigated whether the levels of maternal antibodies could predict a seronegative outcome during follow-up, but found no correlation between the ELISA signal at birth and seroconversion. In addition, we tested the antibody levels in the mothers before and at birth, to investigate whether low antibody titers in the mother could predict seroconversion, but again we found no correlation (data not shown).

The first seroepidemiological study on HCoV-NL63 and HCoV-229E in children was reported by Hofmann et al (10). They determined whether children carry neutralizing antibodies against HCoV-NL63 and HCoV-229E spike (S) glycoprotein. The neutralization assays were performed by challenging pseudo-viruses carrying the HCoV-NL63 or HCoV-229E spike glycoprotein with human sera (10). This resulted in the observation that children until the age of 1.5 years do not carry
neutralizing antibodies against the S glycoproteins from both HCoVs. However, the number of individuals carrying HCoV-NL63 neutralizing antibodies increased in children older than 1.5 years, whereas for HCoV-229E this number remained low (10). We observed however that the number of children with HCoV-NL63 directed antibodies was equal to those with HCoV-229E directed antibodies. Shoa et al also found the same incidence of HCoV-229E and HCoV-NL63 infections (19). They used a part of the C-terminal region of the N protein as antibody capture antigen in an ELISA (19). Antibodies directed to HCoV-NL63 and HCoV-229E were frequently detected in children of 1 year and older. Thus the findings by Shoa et al and our findings are in contradiction with the findings by Hofmann et al. The fact that Hofmann et al measured seropositivity by using the HCoV-229E S protein of the reference strain may explain the difference. For HCoV-229E, it has been determined that the S protein of the reference strain is different from the recent circulating strains (4) and, therefore, it is possible that the reference strain S could not be recognized by HCoV-229E S antibodies that are induced by current circulating strains. The HCoV-NL63 spike used by Hofmann et al was recently obtained (2003) which may explain why HCoV-NL63 S directed antibodies were more frequently found than HCoV-229E S directed antibodies. We also used the HCoV-229E and HCoV-NL63 reference strains to amplify the N gene and express the protein, however, unlike the S protein, the N protein is rather conserved in time (4).

In the past, most of HCoV-229E and HCoV-OC43 serological data and details on clinical manifestations were obtained from infection trials with adult volunteers (1-3, 11, 17). The antibody levels of each volunteer was measured before entering a trial, and all volunteers were HCoV-229E and HCoV-OC43 seropositive, although the antibody levels varied (3). The volunteers with high antibody levels developed no or less clinical symptoms after HCoV-229E or HCoV-OC43 inoculation, whereas those with low antibody levels did develop normal clinical symptoms after inoculation (3). Volunteers could be reinfected with either HCoV-229E or HCoV-OC43, because the antibody titers dropped within one year to the levels before attending the volunteer trials (3). In a similar manner, HCoV-NL63 reinfections might also occur considering the seroepidemiological similarity to HCoV-229E in children. However, this could only be determined by performing trials with volunteers, but, it will raise ethical issues regarding the unraveled pathogenicity of HCoV-NL63. Alternatively, these results could be obtained by monitoring the antibody levels in healthy adults, from which longitudinal collected sera are available. This will allow investigation of the steadiness of HCoV-NL63 directed antibodies, and whether reinfection of HCoV-NL63 occurs.
Acknowledgement

We thank Volker Thiel from the Kantonal Hospital St. Gallen, Switzerland, for the kind gift of the pTRE-HN plasmid. We also thank Margreet Bakker for selecting the longitudinal children sera and Frits Schöler for selecting the cross-sectional children sera. R.D. and L.v.d.H. are supported by VIDI grant 016.066.318 from the Netherlands Organization for Scientific Research (NWO) and by the sixth framework grant LSHM-CT-2006-037276 from the European Union.
Reference List


Figure 1. Lack of cross-reactivity between antibodies directed to HCoV-NL63 N protein and HCoV-229E N protein. Serum of an adult, known to be positive for HCoV-NL63 and HCoV-229E was diluted (1:200) and pre-incubated with serial dilutions of proteins. Panel A. Competition by soluble HCoV-NL63 N protein (closed triangles, continuous line), HCoV-229E N protein (open squares, dashed line) and LacZ protein (open circles, continuous line) on an HCoV-NL63 N protein ELISA. Panel B. Competition by soluble HCoV-NL63 N protein (closed triangles, continuous line), HCoV-229E N protein (open squares, dashed line) and LacZ protein (open circles, continuous line) on an HCoV-229E N protein ELISA. (RLU = relative luminescence units)

Figure 2. HCoV-NL63 and HCoV-229E N protein directed antibody levels in follow-up sera of 13 children. Each graph, A through M, represents the longitudinal profile of HCoV-NL63 N protein (closed triangles, continuous line) and HCoV-229E N protein (open squares, dashed line) directed antibody levels of a single child. The measured antibody levels are indicated as relative luminescence units x10^6 on the Y-axis. The follow-up period is plotted on the X-axis.

Figure 3. Cumulative incidence of HCoV-NL63 and HCoV-229E seroconversion. 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 continuous line, HCoV-229E as a dashed line. The time point of seroconversion was calculated by taking the midpoint between the last seronegative and the first seropositive time point.

Figure 4. Percentage of seropositives to HCoV-NL63 and HCoV-229E in different age groups. The percentage HCoV-NL63 (black bars) and HCoV-229E (grey bars) seropositive individuals was monitored in sera obtained from children of various ages, m = months, y = years.
Figure 1

A

ELISA signal (RLU x 10^6)

B

ELISA signal (RLU x 10^6)

Competitor protein concentrations (µg/ml)
Figure 4

Seropositive (%)