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Detection of human coronaviruses in children with acute gastroenteritis

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ABSTRACT

Background: Human coronaviruses (HCoVs) are known respiratory pathogens. Moreover, coronavirus-like particles have been seen by electron microscope in stools, and SARS-HCoV has been isolated from intestinal tissue and detected in stool samples.
Objectives: To find out if HCoVs can be found in stools of children with acute gastroenteritis and to assess the significance of HCoVs in the etiology of acute gastroenteritis in children.
Study design: 878 stool specimens from children with acute gastroenteritis and 112 from control children were tested by RT-PCR to detect HCoV groups 1B, 2A and SARS. HCoVs were typed by sequencing all PCR positive samples.
Results: Twenty-two (2.5%) of the 878 stool specimens of children with acute gastroenteritis were positive for HCoVs. The following HCoV types were detected: OC43 (10 cases, 45.5%), HKU1 (6 cases, 27.3%), 229E (2 cases, 9.1%) and NL63 (4 cases, 18.2%). In 4 of the cases a HCoV was the only detected virus; in the remaining cases rotavirus or norovirus was found in the same sample. In control groups there were two HCoV positive samples of 112 tested.
Conclusions: This study shows that all known non-SARS HCoVs can be found in stools of children with acute gastroenteritis. On the basis of this study, the significance of coronaviruses as gastrointestinal pathogens in children appears minor, since most of the coronavirus findings were co-infections with known gastroenteritis viruses.

1. Background

The first human coronaviruses (HCoVs) 229E and OC43 were identified in the 1960s.[1–4] These viruses are common causes of upper respiratory tract infections[5,6] but also have association with lower respiratory tract disease especially in patients with underlying disease.[7–9] Coronavirus-like particles have also been seen by electron microscope (EM) in stool samples of both diarrheic and healthy patients evoking discussion about human enteric coronaviruses.[10] The clinical significance of these findings has been unclear and there are findings showing that such putative enteric coronaviruses are antigenically unrelated to OC43 and 229E viruses.[11]

In 2003 interest towards coronaviruses increased when a new coronavirus was found to be a causative agent of SARS (severe acute respiratory syndrome).[12–14] SARS-HCoV caused a serious lower respiratory tract infection with high mortality.[15–17] The main symptoms were fever, chills, myalgia, cough and headache, but also diarrhea was common and in one study registered in 38.4% of patients. In the same study SARS-HCoV was also isolated from intestinal tissue and viral RNA was found in 16% of stool samples, which was comparable to the detection rate in nasopharyngeal aspirates.[18] In another cohort of patients diarrhea was seen in 73% of patients and viral RNA was detected in 97% of stool specimens. In this cohort it was suggested that the outbreak was caused by faulty sewage system whereby the transmission might been fecal–oral rather than respiratory.[19] No association between the presence of diarrhea and mortality in SARS cases has been observed, however.[20]

A fourth human coronavirus, HCoV-NL63, was identified in 2004 in the Netherlands. It was isolated from a 7-month-old child suffering from bronchiolitis and conjunctivitis, and was categorized to be a new group 1 coronavirus.[21] Soon after, another group in the Netherlands independently detected the same virus in an 8-month-old child with pneumonia.[22] Since the discovery, NL63 has been detected in patients with respiratory tract infection in several countries around the world, including Canada, Australia, Korea and France.[23–26] NL63 has been associated with severe lower respiratory tract infection and croup.[24,27] The seroprevalence in 6–12-month-old children for NL63 is 28.6–40.0%.[28]

Less than a year later a second new coronavirus, HCoV-HKU1, was discovered in Hong Kong. This group 2 coronavirus was detected in a 71-year-old man with pneumonia.[29]

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HKU1 has been found globally[30–33] and in addition to respiratory samples it has been detected in stools, but no clear connection to enteric disease has been found.[34]

2. Objectives

Considering the EM findings, presence of gastrointestinal symptoms in SARS, and the findings of HKU1 in stools, we wanted to find out if non-SARS human coronaviruses could be detected in stool samples of children with acute gastroenteritis using RT-PCR assay in order to assess the potential significance of coronaviruses in the etiology of acute gastroenteritis in children.

3. Study design

The clinical material for the study was collected in a prospective study of acute gastroenteritis in children Tampere and Kuopio University Hospital during a 2-year period 2006–2008 (Räsänen et al., unpublished). Healthy children ($N = 36$), children with indeterminate fever and vomiting ($N = 43$), and children with respiratory tract infection ($N = 33$) were used as control groups. Also group A rotavirus, calicivirus (including norovirus genogroups I and II, and sapovirus), aichivirus and human bocavirus were studied from the same material. Adenovirus was not tested systematically but of 101 samples tested 13 were positive for adenovirus (six belonging to subgroup F, types 40 and 41) and none of these were co-infections with coronaviruses including two positive samples for coronavirus. A total of 878 samples from children with acute gastroenteritis, 43 samples from children with indeterminate fever and vomiting, 33 samples from children with respiratory tract infection and 36 samples from healthy children were tested for HCoVs.

4. Laboratory methods

Before RNA extraction the stool samples were diluted in phosphate-buffered saline (PBS) creating 10% stool suspension. Viral RNA was extracted using QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. Extracted RNA was stored at −70 °C until used.

Reverse transcription was done using random primers as previously described by Pang et al. (2005)[35] except that reaction contained 1× First Strand Buffer (Invitrogen, USA) and the final concentration of dNTPs (Promega, USA) was 375 nM per each nucleotide. The produced cDNA was stored at −20 °C.

We used a two-step nested PCR which was set up and optimized in our laboratory. Primers targeted to polymerase gene region were chosen because of regions conserved nature. HCoV strains TC-adapted OC43 (VR-1558) and 229E (VR-740) were purchased from ATCC (USA), and propagated in HCT-8 and MRC-5 cells, respectively. In addition, positive HKU1 and NL63 samples were provided by Professor Tobias Allander (Karolinska University, Sweden) and Professor Lia van der Hoek (University of Amsterdam, Netherlands). The specificity of PCR was confirmed by testing 35 different respiratory or enteric viruses and bacteria, with negative results.

The 1st PCR included a primer pair (fwd-GWTGGGAYTATCCNAARTGTGA and rev-YRTCATCASWNARAATCATCAT) universal for all coronaviruses with resulting amplicon of 437 bp in size. 10 μl of the cDNA was added to 40 μl of reaction mixture containing 1× Green GoTaq® Flexi Buffer (Promega, USA), 2.5 mM of GoTaq® $MgCl_2$ (Promega, USA), 200 μM of each dNTP (Promega, USA), 2.5 U of GoTaq DNA polymerase (Promega, USA) and 0.5 μM of each primer (Sigma-Genosys Ltd., UK). PCR program was run as follows: 2 min at 94 °C, 35 cycles of amplification (30 s at 94 °C, 30 s at 54 °C, 1 min at 72 °C) and final extension at 72 °C for 5 min. Program was run in GeneAmp® PCR system 9700 (PE Applied Biosystems, USA) or in Thermal Cycler 2720 (Applied Biosystems, USA). Aqua Sterilisata H_2O was used as negative control and cell cultured 229E or OC43 as a positive control.

Nested PCR contained three primer pairs to distinguish three coronavirus groups: 1B, 2A and SARS. Sizes for the amplicons were 203 bp for group 1B (fwd-GTTGTTTATTCWAATGGTGG and rev-YCTATARCAATTATCATAMAG), 275 bp for group 2A (fwd-WYTRCGTATTGTTAGTAGTTTRGT and rev-CGTATACTWARATCTTCAATCTT) and 230 bp for SARS (fwd-TGCTGTAACTTATCACACCGT and rev-CGGACATACTTGTCAGCTATCT). 2 μl of 1st PCR-product was added to 48 μl of reaction mixture containing 1× Green GoTaq® Flexi Buffer (Promega, USA), 1.5 mM of GoTaq® $MgCl_2$ (Promega, USA), 200 μM of each dNTP (Promega, USA), 2.5 U of GoTaq DNA polymerase (Promega, USA) and 0.5 μM of each primer (Sigma-Genosys Ltd., UK). PCR program was run as follows: 2 min at 94 °C, 35 cycles of amplification (30 s at 94 °C, 30 s at 53 °C, 30 s at 72 °C) and final extension at 72 °C for 5 min.

PCR-products were separated and recognized by gel electrophoresis, and all positive PCR products were confirmed to be coronaviruses and the specific type defined by sequencing. ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, USA) was used in sequencing. Sequences were aligned and confirmed by using Sequencher™ 4.8 program (Gene Codes Corporation, USA) and confirmed sequences were compared to reference strains by NCBI Blast®-program.

5. Results

Twenty-two (2.5%) of the 878 stool specimens of children with acute gastroenteritis were positive for HCoVs. A HCoV as a single pathogen was detected in only four of the samples (18.2% of the positive samples). In the remaining cases either norovirus or rotavirus was detected in the same sample (Table 1). In eleven (50%) of the 22 coronavirus positive cases there were symptoms of respiratory tract infection at the same time with gastroenteritis, or respiratory symptoms had been present before symptoms of gastroenteritis.

Three of the 4 patients with coronavirus as a single pathogen in stool sample had respiratory tract related symptoms. Patient 1 (Table 1) had cough and rhinitis, and had just recovered from otitis media. Patient 7 (Table 1) did not have any respiratory tract symp-

Table 1
Positive detections of human coronaviruses in 878 children with acute gastroenteritis.

Patient	Age (months)	Sex	Sample date	HCoV type	Other virus	Respiratory symptoms[a]
1	17	F	January 2007	OC43	None	Yes
2	27	M	February 2007	OC43	noro	None
3	12	F	February 2007	OC43	noro	Yes
4	41	F	February 2007	OC43	noro	None
5	13	F	March 2007	OC43	noro	Yes
6	27	F	March 2007	NL63	rota	None
7	17	M	April 2007	NL63	None	None
8	25	M	February 2007	NL63	noro	None
9	13	M	April 2007	OC43	noro	Yes
10	9	M	March 2007	OC43	noro	None
11	31	F	June 2007	NL63	None	Yes
12	45	M	November 2007	OC43	rota	Yes
13	27	F	December 2007	OC43	rota	Yes
14	18	M	January 2008	229E	rota	None
15	17	M	January 2008	HKU1	noro	Yes
16	29	M	February 2008	OC43	rota	None
17	17	F	February 2008	HKU1	noro	None
18	10	M	February 2008	HKU1	rota	None
19	75	M	March 2008	HKU1	None	Yes
20	21	M	April 2008	HKU1	rota	Yes
21	30	M	April 2008	229E	rota	Yes
22	16	F	April 2008	HKU1	rota	None

[a] Including cough, rhinitis, tonsillitis, otitis media, pneumonia, laryngitis.

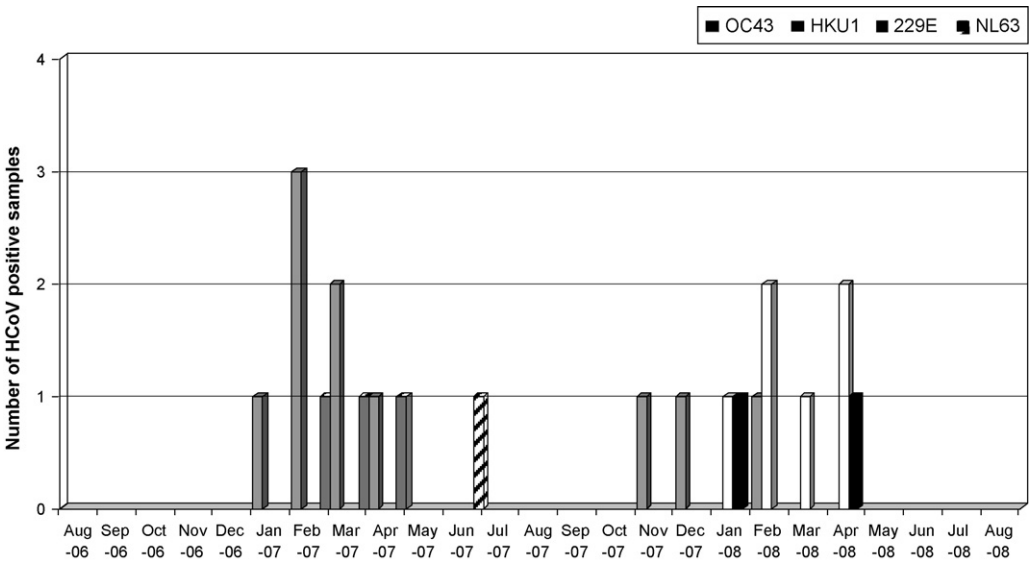

Fig. 1. Seasonal distribution of human coronavirus positive stool specimens of children with acute gastroenteritis.

toms. Patient 11 (Table 1) had tonsillitis and patient 19 (Table 1) had headache and dizziness in addition to symptoms of respiratory tract infection.

All non-SARS human coronavirus types were found, members of group 2A; OC43 (10 of the cases, 45.5%) and HKU1 (6 of the cases, 27.3%) were most common, whereas group 1B viruses 229E and NL63 were found only in 2 and 4 cases, respectively. No SARS or SARS-like viruses were found. Still there might be unknown coronaviruses that our PCR method did not detect in spite of the universal primers in the 1st PCR. Most HCoV positive cases were found from January to April (Fig. 1).

The age distribution of the coronavirus infected children was 9–75 months (median, 19.5 months), whereas in the total material the youngest child was 14 days and the oldest 14 years and 4 months (median, 17 months). Of the coronavirus positive children 59% were males.

Within the control groups two (1.8%) of the 112 stool samples were positive for HCoV. One of the cases was a 3-year-old female with pneumonia. OC43 was detected as the only pathogen in her stool samples on February 2007. Two days after sample collection she also developed symptoms of gastroenteritis. The second patient was a healthy female aged 2 years and 11 months tested on July 2007. OC43 was again detected in a stool sample as a single pathogen.

6. Discussion

Our study shows that human coronaviruses OC43, HKU1, 229E and NL63 can be found in stool samples of children with acute gastroenteritis. The significance of coronaviruses as gastrointestinal pathogens seems at most marginal, even though it is also possible that our PCR method did not detect all existing coronaviruses and there still might be unknown coronaviruses related to diarrheal disease. Most of the coronavirus findings were co-infections with well known enteric pathogens, norovirus and rotavirus. Furthermore, half of the patients with coronavirus in stools had symptoms related to respiratory tract infection and, therefore, HCoVs found in the stools could have originated from respiratory tract. Unfortunately, no specimens from respiratory tract were collected to confirm the presence of coronaviruses in the respiratory tract in these patients, and further studies are needed to evaluate simultaneous presence of HCoV in stools and respiratory tract. Even if coronaviruses were found in respiratory tract in cases of acute gastroenteritis it would be difficult to determine whether they were primarily causing the respiratory or gastrointestinal symptom.

Studies with SARS have shown that RNA of SARS coronavirus can be detected in stool samples for more than 10 weeks after symptom onset.[18] This elicits the question whether also non-SARS coronaviruses might be detected after a prolonged time from the original infection. Previous studies with EM showed that coronavirus-like particles can be seen in stools of both diarrheic and healthy patients.[10] In our study one of the 36 healthy control patients had coronavirus detected in stool specimen and thus, there was no difference in the HCoV detection rate between the cases of acute gastroenteritis and control children. This study was hospital based and did not include mild cases of gastroenteritis treated at home or healthcare centers. Future studies should investigate such mild cases for HCoVs.

In conclusion, non-SARS human coronaviruses can be found in stool samples of children with acute gastroenteritis. However, such findings are rare and occur usually with other well established gastroenteritis viruses. HCoVs may also be found in occasional stool samples of children without gastroenteritis. Taken together, it appears that known HCoVs may at most have a minor etiologic role in the acute gastroenteritis of children.

Ethical approval

The study protocol and consent forms had been approved by the Ethics Committee of the Pirkanmaa Hospital District in 2006.

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None.

Conflict of interest

None declared.

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