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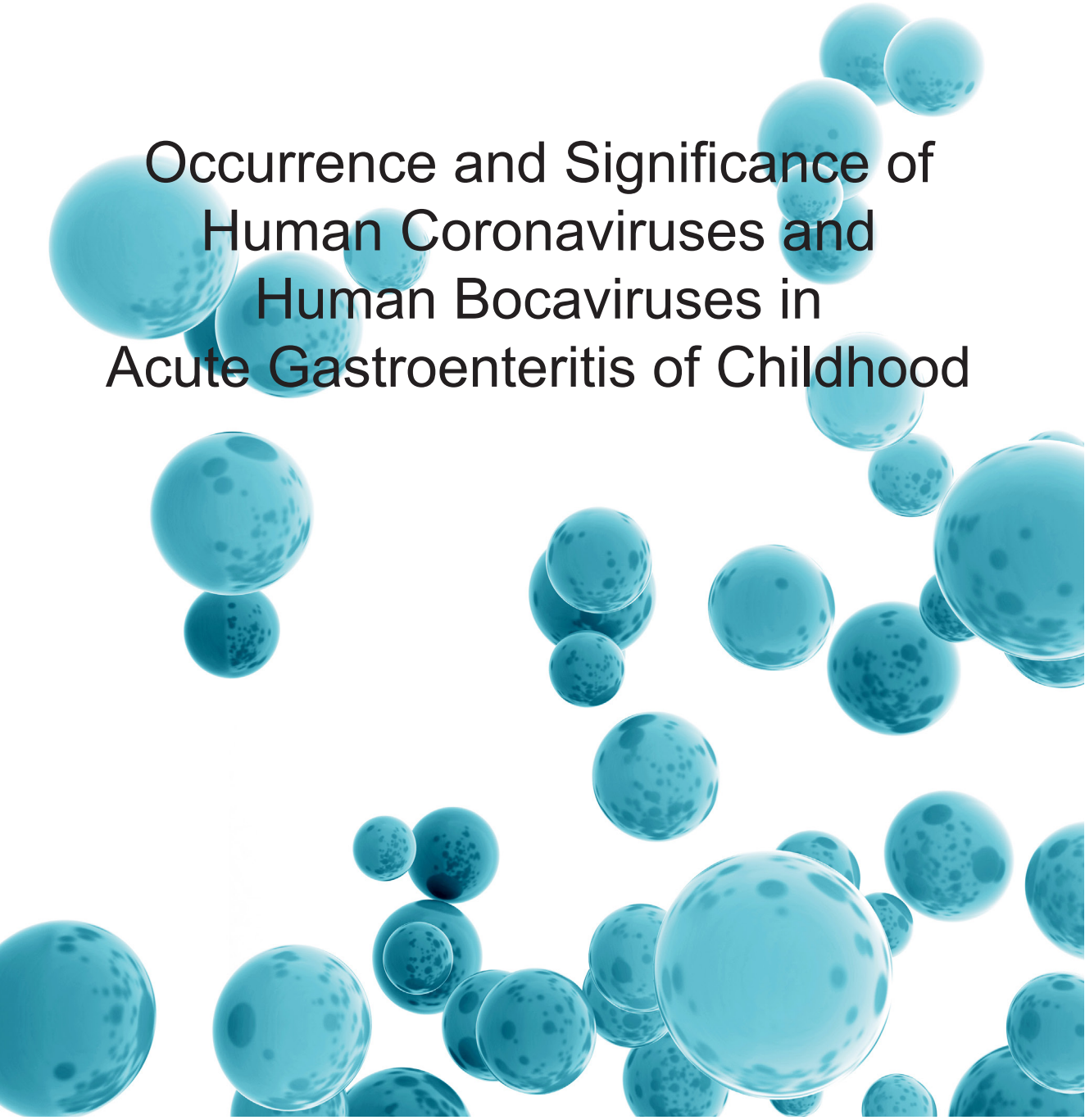
Occurrence and Significance of Human Coronaviruses and Human Bocaviruses in Acute Gastroenteritis of Childhood



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Occurrence and Significance of Human Coronaviruses and Human Bocaviruses in Acute Gastroenteritis of Childhood

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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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To my family,

Abstract

Acute gastroenteritis (AGE) remains an important cause of child hospitalization in Finland, although rotavirus vaccination has reduced the number of AGE hospitalizations significantly. After the elimination of rotaviruses, noroviruses – and to a smaller extent adenoviruses – are the leading viral causes of the AGE in children. Despite the use of comprehensive diagnostic methods, there are still cases of AGE in children without a known causative pathogen.

In recent years, new viruses, such as human bocaviruses (HBoVs) and human coronavirus (HCoV) types NL63 and HKU1, have been discovered. There has been suggestive evidence that these new viruses might be associated with AGE, warranting further studies.

The aim of this dissertation was to investigate the occurrence of HCoVs and HBoVs in children hospitalized for AGE and to see if the virus detections in stools are associated with AGE. Two large datasets of prospectively collected patient materials were studied; they were collected at Tampere University Hospital in 2006-2008 and 2009-2011. Stool samples were available from the first group, and stool, nasal swab, and serum samples were available from the second group.

A reverse transcription-polymerase chain reaction (RT-PCR) method designed to detect all known HCoV types was set up. Before this study, only SARS (severe acute respiratory syndrome) CoV had been studied systematically from stool samples by molecular techniques. In the first study, we found that all non-SARS HCoVs – including OC43, 229E, and the new types NL63 and HKU1 – were present in some stool samples of children hospitalized for AGE, but the overall detection rate was low, 2.5%. In addition, 1.8% of the control children without gastroenteritis harbored HCoVs in their stools, and several of the HCoV-positive children with AGE also had respiratory symptoms. In the second study, we studied both the stool and nasal swab samples of children with AGE and also the stool and nasal swab samples of children hospitalized for acute respiratory tract infection (ARTI). Some of the children had symptoms of both AGE and ARTI. This second study revealed that in addition to children with AGE, children with ARTI – and children with symptoms of both AGE and ARTI – also harbored HCoVs in their stools. Furthermore, if HCoV was found in the stool sample, in 90% of the cases it was found in the nasal

swab sample as well. In addition, if an HCoV was found in children with AGE, in most cases there was a known gastroenteritis virus, usually rotavirus or norovirus, in the same stool sample. Combining the results of these two studies, it was concluded that commonly circulating HCoVs (OC43, 229E, NL63, and HKU1) are not significant causes of AGE in hospitalized children. Instead, it seems that a positive stool sample is associated with positivity in the nasal swab sample, and thus a finding of HCoV in the stool is rather a consequence of acute or recent respiratory tract infection.

HBoVs were studied by PCR, and in the third study, it was found that HBoV types 1, 2, and 3 were found in 5.6%, 3.3% and 0.9% of the stool samples of children with AGE respectively, but they were usually found together with known gastroenteritis viruses. In addition, some of the control children without symptoms of AGE harbored HBoVs. In the fourth study, both PCR and enzyme immunoassays (EIAs) were used to detect HBoV DNA and HBoV-specific antibodies, respectively. HBoVs from both stool and nasal swab samples were studied in a similar way to the HCoVs, and it was found that HBoV2 and 3 were mainly detected in stools, whereas HBoV1 was commonly found in both stool and nasal swab samples. HBoV1 findings were associated with symptoms of respiratory tract infections, in line with being a respiratory virus, while HBoV2 and HBoV3 were found at similar rates in children with and without AGE. In over 90% of the HBoV-positive stool samples of children with AGE, a well-established gastroenteritis virus was detected in the same sample. Even in the cases of acute HBoV2 infections, norovirus was simultaneously detected in the stools, thus being the most probable reason for the symptoms.

In conclusion, neither HCoVs nor HBoVs were associated with the children's AGE in this study. HCoVs are principally respiratory viruses and findings in stools are probably remnants of the infection in the respiratory tract. HBoV2 and 3 are mainly found in stool samples, and may be regarded as enteric viruses, but no association with AGE in the hospitalized children was found.

Tiivistelmä

Akuutti gastroenteriitti eli äkillinen maha-suolitulehdus on edelleen tärkeimpiä syitä lasten sairaalahoitoon Suomessa, vaikka rotavirusrokotteen otto kansalliseen rokotusohjelmaan on enemmän kuin puolittanut sairaalahoitojen määrän. Rotavirusten eliminoinnin jälkeen norovirukset, ja jossain määrin enteeriset adenovirukset, ovat tulleet tärkeimmiksi lasten virusperäisen gastroenteriitin aiheuttajiksi Suomessa. Diagnostiikan kehittymisestä huolimatta kuitenkin osassa lasten gastroenteriittitapauksista taudinaiheuttaja jää tunnistamatta.

Viime vuosina on löydetty useita uusia viruksia, kuten ihmisen bokavirukset ja ihmisen koronavirustyypit NL63 ja HKU1. Näitä uusia viruksia on löydetty myös ulostenäytteistä.

Tässä väitöskirjatyössä selvitettiin ihmisen koronavirusten ja ihmisen bokavirusten esiintymistä sairaalahoitoon joutuneiden lasten ulosteissa sekä näiden virusten yhteyttä akuuttiin gastroenteriittiin. Tutkimuksessa käytettiin kahta Tampereen yliopistollisessa sairaalassa vuosina 2006-2008 ja 2009-2011 kerättyä aineistoa, joista ensimmäinen sisälsi vain ulostenäytteitä, ja toinen ulostenäytteitä, hengitystienäytteitä ja verinäytteitä.

Ihmisen koronavirusten yhteyttä gastroenteriitteihin tutkittiin käänteiskopiointi polymeraasi ketjureaktio (RT-PCR) – menetelmällä, joka suunniteltiin tunnistamaan kaikki tunnetut ihmisen koronavirustyypit. Ennen tätä tutkimusta ainoastaan SARS-koronaviruksen esiintymistä ulosteissa oli tutkittu systemaattisesti. Ensimmäisessä osatyössä ilmeni, että kaikkia yleisesti kiertäviä koronavirustyyppejä, OC43, 229E, NL63 ja HKU1, löytyi gastroenteriittiin sairastuneiden lasten ulosteista, mutta koronaviruspositiivisten näytteiden osuus tautitapauksista oli pieni, kokonaisuudessaan vain 2,5 %. Lisäksi joillakin kontrolliryhmän lapsilla, joilla ei ollut gastroenteriittiä, löytyi koronavirus-positiivisia ulostenäytteitä ja osalla gastroenteriittiä sairastavista koronavirus-positiivisista lapsista oli myös hengitystieinfektion oireita. Toisessa osatyössä koronavirusten esiintymistä tutkittiin ulostenäytteiden lisäksi saman tautiepisodin aikana nenän limakalvolta otetuista tikkunäytteistä. Näytteitä tutkittiin sekä gastroenteriitin että hengitystieinfektion takia sairaalahoitoon joutuneilta lapsilta. Osalla lapsista esiintyi yhtä aikaa sekä hengitystieinfektion että gastroenteriitin oireita. Toisessa osatyössä selvisi, että

koronaviruksen löytyessä ulosteesta myös hengitystienäyte oli 90 % tapauksista positiivinen. Koronaviruksia löytyi sekä hengitystieinfektiopotilaiden että gastroenteriittiin sairastuneiden potilaiden ulosteista ja gastroenteriittitapauksissa ulosteesta löytyi yleensä myös jokin tunnettu gastroenteriittia aiheuttava virus, kuten rotavirus tai norovirus. Näiden kahden osatyön perusteella vaikuttaa siltä etteivät yleisesti kiertävät koronavirustyypit OC43, 229E, NL63 ja HKU1 ole lasten sairaalahoitoa vaativien gastroenteriittien aiheuttajia vaan positiivinen ulostenäyte liittyy viruksen läsnäoloon hengitysteissä ja on siten pikemminkin merkki akuutista tai aiemmin sairastetusta hengitystieinfektiosta kuin gastroenteriitistä.

Ihmisen bokavirusten tutkimiseen käytettiin PCR-menetelmää ja neljännessä osatyössä myös ihmisen bokaviruksille spesifien vasta-aineiden tunnistamiseen EIA-menetelmää. Väitöskirjan kolmannessa osatyössä todettiin bokavirustyyppien 1, 2 ja 3 esiintyvän gastroenteriittiin sairastuneiden lasten ulostenäytteissä, mutta useimmiten yhdessä jonkin tunnetun gastroenteriittiviruksen kanssa. Lisäksi myös osalla kontrolliryhmän lapsista löytyi bokaviruspositiivisia ulostenäytteitä. Neljännessä osatyössä bokaviruksia tutkittiin koronavirusten tapaan ulostenäytteiden lisäksi myös hengitystienäytteistä ja voitiin todeta, että bokavirustyypit 2 ja 3 löytyvät pääasiassa ulostenäytteistä, kun taas tyyppi 1 esiintyy ulostenäytteiden lisäksi myös hengitystienäytteissä. Eri tutkimusryhmiä vertailemalla voitiin todeta, että ihmisen bokavirus 1:n esiintyminen näytteissä liittyy hengitystieinfektioihin, mutta ihmisen bokavirus 2:n ja ihmisen bokavirus 3:n esiintyminen ei eronnut tutkimusryhmien välillä merkittävästi. Lisäksi gastroenteriittitapauksissa ulosteesta löytyi yli 90% tapauksista bokaviruksen lisäksi jokin tunnettu gastroenteriittiä aiheuttava virus, joka todennäköisimmin oli oireiden aiheuttaja.

Väitöskirjatyön perusteella mikään tutkituista viruksista ei lunastanut paikkaansa lasten sairaalahoitoa vaativan gastroenteriitin aiheuttajana. Koronavirukset ovat ensisijaisesti hengitystieinfektioita aiheuttavia viruksia ja ulostelöydökset liittyvät yleensä viruksen samanaikaiseen esiintymiseen hengitysteissä. Bokaviruksista tyypit 2 ja 3 ovat pääasiassa suolistossa viihtyviä viruksia, mutta niiden yhteyttä lasten sairaalahoitoiseen gastroenteriittiin ei pystytty osoittamaan.

List of Original Communications

This thesis is based on the following publications, which are referred to in the text by Roman numerals as listed below. The original articles are reprinted with the permission of the copyright holders.

I. Detection of human coronaviruses in children with acute gastroenteritis.
Minna Risku, Suvi Lappalainen, Sirpa Räsänen, Timo Vesikari. Journal of Clinical Virology 2010 May;48(1):27-30.

II. Commonly circulating human coronaviruses do not have a significant role in the etiology of gastrointestinal infections in hospitalized children.
Minna Paloniemi, Suvi Lappalainen, Timo Vesikari. Journal of Clinical Virology 2015 Jan;62:114-7.

III. Human bocavirus types 1, 2 and 3 in acute gastroenteritis of childhood.
Minna Risku, Minna Kätkä, Suvi Lappalainen, Sirpa Räsänen, Timo Vesikari. Acta Paediatrica 2012 Sep;101(9): pp.e405-e410.

IV. Human bocaviruses are commonly found in stools of hospitalized children without causal association to acute gastroenteritis.
Minna Paloniemi, Suvi Lappalainen, Marjo Salminen, Minna Kätkä, Kalle Kantola, Lea Hedman, Klaus Hedman, Maria Söderlund-Venermo, Timo Vesikari. European Journal of Pediatrics 2014 Aug;173(8):1051-7.

Abbreviations

ACE-2	angiotensin converting enzyme 2
AGE	acute gastroenteritis
ARTI	acute respiratory tract infection
BCV	bovine coronavirus
Bp	base pair
BPV	bovine parvovirus
cDNA	complementary DNA
CoV	coronavirus
CSF	cerebrospinal fluid
DDP4	dipeptidyl peptidase 4
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ER	emergency room
gRNA	genomic RNA
HAE	human airway epithelium
HBoV	human bocavirus
HCoV	human coronavirus
IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
Kb	kilobase
MERS	Middle East respiratory syndrome
MHV	mouse hepatitis virus

MMP matrix metalloprotease

mRNA messenger RNA

MVC minute virus of canines

NEC necrotizing enterocolitis

OAS original antigenic sin

ORF open reading frame

PBS phosphate-buffered saline

PBSP PBS+0.05% polysorbate 20

PCR polymerase chain reaction

RT-PCR reverse transcription polymerase chain reaction

RTC replication-transcription complex

SARS severe acute respiratory syndrome

VLP virus-like particle

Contents

1 Introduction

Acute gastroenteritis (AGE) remains an important cause of childhood morbidity and mortality in both developed and developing countries. Globally, approximately 10% (800,000) of deaths in children under five years of age are due to diarrhea, even though this number has decreased over the last ten years (1).

More than 20 viral, bacterial, and parasitic pathogens are associated with acute gastroenteritis, but some of them still require confirmation of their role. In countries with good sanitary conditions, like most European countries, viruses are the principal cause of AGE in children (2).

It has been estimated that in 2008, rotavirus caused the deaths of 453,000 children under five years of age worldwide, with most of the deaths occurring in developing countries (3). There are also estimates that 3.6 million episodes of rotavirus infection occur annually in children in the European Union, leading to 231 deaths and over 87,000 hospitalizations (4). Rotavirus is the most common cause of hospitalizations due to AGE in Europe (5). Before the national rotavirus vaccination program was initiated in 2009, rotaviruses were responsible for 38-63% of AGE cases in children requiring hospital care in Finland (6). After universal vaccinations, the number of hospitalizations due to rotavirus gastroenteritis has decreased by over 70%, and now norovirus has become the leading cause for AGE in hospitalized children in Finland, even though the absolute number of norovirus cases has not increased (7). Likewise in Finland, norovirus has recently overtaken rotavirus as the leading cause of AGE in children in other countries that have a universal rotavirus vaccination program (8,9).

In addition to rotavirus and norovirus, three other significant gastroenteritis viruses are recognized in children. Sapovirus is a member of the *Caliciviridae* family – like norovirus – but detection rates are lower (7,9,10) and clinical picture milder compared to norovirus (11,12). Astrovirus has been detected in approximately 10% (11) of children with AGE in the community, but the symptoms are usually mild and the detection rates in hospitalized children have been lower (11,13). Adenovirus (enteric types 40 and 41) has been, after rota- and norovirus, the third most common viral pathogen in hospitalized children with AGE (11).

Despite the improved diagnostic methods, there still is a significant group of children with the symptoms of AGE but in whom none of the above-mentioned well-known enteric viruses are detected. This "diagnostic gap" has varied between 20% and 40% of AGE cases, depending on the study design and methods used (11,13,14). In the study materials used in this dissertation, approximately 70% of children hospitalized for AGE had rotavirus or calicivirus (norovirus or sapovirus) in their stool sample (7); adenovirus and astrovirus together covered approximately 18% of the cases (unpublished).

As a result of the recent advances in molecular techniques, several new viruses have been found in stool samples, increasing hopes of finding the pathogens that would fill "the diagnostic gap" in AGE in children. Human coronavirus types NL63 (15) and HKU1 (16), and human bocaviruses (17-20) belong to these interesting, recently discovered viruses. In the following literature review, human coronaviruses and human bocaviruses are introduced and the background for this dissertation is provided.

2 Review of the Literature

2.1 Human coronaviruses

2.1.1 Classification

Human coronaviruses are members of the family *Coronaviridae*, subfamily *Coronavirinae*. The subfamily is formed of three genera: *Alphacoronavirus, Betacoronavirus*, and *Gammacoronavirus*. The genus *Betacoronavirus* is further divided into four separate lineages (A-D). Recently, the formation of a fourth genus, *Deltacoronavirus*, has been proposed. The members of the family are assigned to a subfamily and genus according to the rooted phylogeny and calculation of pair-wise evolutionary distances in seven conserved regions of the replicase polyprotein pp1ab. Viruses with a shared amino acid sequence identity of over 90% in the conserved regions are considered to belong to the same species (21).

HCoVs belong to the genera *Alphacoronavirus* (229E and NL63), *Betacoronavirus* lineage A (OC43 and HKU1), *Betacoronavirus* lineage B (SARS), and *Betacoronavirus* lineage C (MERS) (21,22). In addition to human coronaviruses, the subfamily *Coronavirinae* includes several coronaviruses of other mammals and birds (21).

Another subfamily of the family *Coronaviridae* is *Torovirinae*, which includes two genera, *Torovirus* and *Bafinivirus* (21).

2.1.2 Structure

Coronaviruses are enveloped viruses with a helical nucleocapsid and positive-sense single-stranded RNA. In negative-staining electron microscopy (EM), virus particles appear in multiform, roughly spherical shapes with characteristic surface projections that give the virus its crown-like appearance. The diameter of the virion varies from 120 to 160 nm (see Figure 1) (21).

Coronaviruses have four main structural proteins (S, M, E, and N) that are present in all species, and additional structural accessory proteins that depend on the

particular coronavirus species; these latter proteins have various functions (21,23). The spike protein S is the most important determinant of cell tropism and host range; it forms the surface projections of the virion and mediates receptor binding and membrane fusion (21,24). Membrane protein M is part of the envelope and has an important role in the assembly and structure of the envelope (25). The envelope protein E is multifunctional: it is involved in virion assembly and morphogenesis, (21) and it is, for example, an important factor for the pathogenesis and virulence of SARS-CoV due to its ion channel activity (26). Nucleocapsid protein N is also a multifunctional protein. In addition to its primary role in genome encapsidation, it is involved in viral assembly, envelope formation, and genomic replication; it also influences host cell functions in many ways (27).

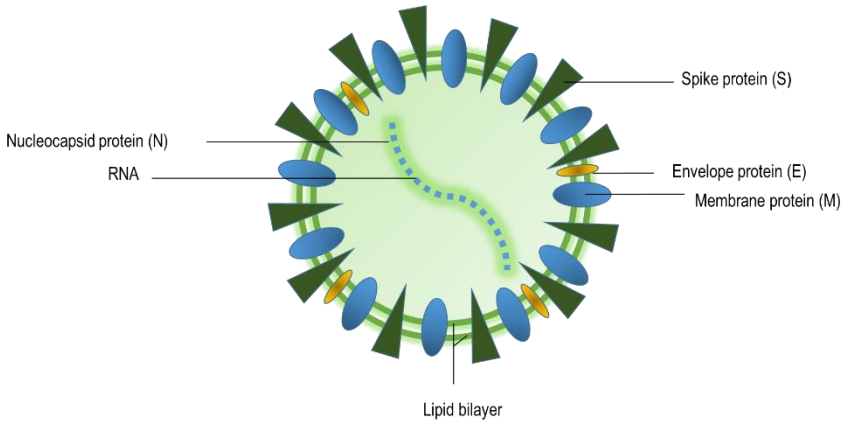


Figure 1. Schematic presentation of the structure of a coronavirus particle. (Modified from 28,29)

2.1.3 Genome

At 26-32 kb in length, the genomes of coronaviruses are known to be the largest and the most complex of the RNA viruses. There are six open reading frames (ORFs) that are common to all members of the subfamily, and they are arranged in the following order from the 5' end to the 3' end: ORFs 1a and 1b (which together form the replicase gene that encodes several non-structural proteins), followed by ORFs for S, E, M, and N (the structural proteins). In addition, there are usually several

species- or strain-specific accessory genes between the ORFs of the common genes (Figure 2) (21,30).

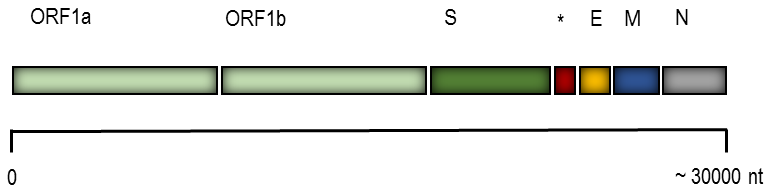


Figure 2. Schematic representation of the genome organization of coronaviruses. Boxes represent different genes. All coronaviruses share the basic organization of the genome, but the location of accessory genes (marked as *) may vary. (Modified from 29)

2.1.4 Replication cycle

2.1.4.1 Virus entry

The spike protein S plays a key role in both the receptor binding and the fusion of the host and viral membranes. The S protein can be divided into two main domains: S1 and S2. S1 is responsible for receptor binding and its sequence can vary significantly, whereas S2 leads the fusion process and has a relatively conserved sequence. After binding to a specific receptor, major conformational changes occur in the S protein and initiate the fusion. Fusion can take place directly at the cell surface after receptor binding or following the endocytosis. Usually productive fusion requires additional factors, like endosomal proteases, in addition to receptor binding. The specific mechanisms of coronavirus entry are complicated and vary between the different coronavirus species and strains (24).

2.1.4.2 Replication and transcription

The replication and transcription processes of coronaviruses occur entirely in the cell cytoplasm (21). The novel RNA is produced in the so-called replication-transcription complexes (RTCs), which contain viral and also possibly cellular proteins associated with intracellular membranes. Studies have suggested that most of the non-structural proteins of coronaviruses are involved in the activity of

these RTCs. At the beginning of the replication cycle coronavirus genomic RNA (gRNA) acts directly as messenger RNA (mRNA) to product replicase polyproteins. Replicase polyproteins are then processed by virus-derived proteases into several shorter proteins that form part of the RTCs. Following the formation of the replicase proteins, the positive-sense gRNA is copied into negative-sense counterparts that serve as templates for the production of new gRNAs. In addition to a negative-sense gRNA, coronaviruses produce several shorter negative-sense counterparts of subgenomic mRNAs. A specific feature of the subgenomic mRNAs is that they contain sequences corresponding to both ends of the genome, and the formation is involved in the process called discontinuous transcription. However, this transcription model is not entirely understood (30,31,32).

Like most RNA viruses, coronaviruses mutate fast because the RNA polymerase enzyme produces errors in high frequency. In addition, RNA recombination events are common, which may be a consequence of the discontinuous transcription process. (32)

2.1.4.3 Assembly and release of virions

It has been suggested that the M and E proteins, or their complexes, determine the site of virus assembly and budding. The formation of the nucleocapsid involves the interaction between N proteins and the viral RNA, and the developed nucleocapsid further interacts with M proteins, and the budding of the nucleocapsid through the membranes of the endoplasmic reticulum and Golgi complex follows. Finally, the mature virions are excreted from the cell by exocytosis (32). Coronavirus virions are released from their main target cells using two principal routes: apical or basolateral release. This pattern of release may partly explain the pathogenesis of certain coronaviruses, as will be discussed later (33).

2.1.5 Pathogenesis

The first step in the pathogenesis of viruses is to come into contact with a susceptible cell, i.e. a cell with a suitable receptor. Coronaviruses use a wide variety of cell surface molecules as receptors, and receptor usage among the different HCoVs varies. As explained above, the spike protein S is the major receptor binding component of

coronaviruses, and for this reason it is mainly responsible for the cell tropism of different coronaviruses (24).

HCoV-229E uses aminopeptidase N, a metalloprotease located on the surface of intestinal, lung, and kidney epithelial cells, as a receptor (34). Both SARS-CoV and HCoV-NL63 use angiotensin converting enzyme 2 (ACE-2) as a receptor (35,36), but they probably use different strategies when binding to the ACE-2 molecule (36). The ACE-2 gene is expressed widely in human tissues, including the lungs, but the highest levels of expression have been detected in gastrointestinal, renal, and cardiovascular tissues (37). SARS-CoV can also use CD209L (L-SIGN) glycoprotein as an additional receptor, but it is less efficient as a receptor than ACE-2 (38). N-acetyl-9-O-acetylneuraminic acid and HCoV-OC43 have been linked, but a definite receptor for OC43 cell entry has not been confirmed (39). The major histocompatibility complex class I C molecule facilitates HCoV-HKU1 attachment to the cell surface but other – currently unknown – receptors are needed for cell entry (40). Recently identified MERS (Middle East respiratory syndrome)-CoV uses dipeptidyl peptidase 4 (DDP4) as a functional receptor for cell entry. DDP4 is a transmembrane glycoprotein that is expressed, for example, in the epithelial cells of the kidney, small intestine, and liver, and in non-ciliated bronchial epithelial cells. (41) A comprehensive study with the human airway epithelium (HAE) revealed that HCoV-OC43, HCoV-HKU1, and HCoV-NL63 mainly infect the ciliated epithelial cells, but the primary target cells of HCoV-229E in the HAE were nonciliated (42).

When exploring the location of cells with suitable receptors for HCoVs and the clinical picture of HCoV infections – which are most commonly in the respiratory tract – it seems that epithelial cells in the respiratory tract, and possibly in the intestinal tract, are likely the first targets of HCoVs. These epithelial cells lining the respiratory and intestinal cavities are functionally polarized, meaning that they have two functionally and compositionally distinguishable regions on their surfaces: the apical and basolateral (43). The way that HCoV interacts with these cells, especially the method of virus release, may have a great influence in the outcome of the infection. Usually, HCoVs enter the epithelial cell by its apical membrane, which is the region facing the lumen of the cavity, for example the surface of the upper respiratory tract. If the release of the virus also takes place in the apical surface – as in the case of HCoVs OC43, 229E, NL63, HKU1, and SARS – most of the reproduced viruses are concentrated on the surface of the respective cavity, hence more likely resulting in an infection restricted to the epithelial surface (33,42). A massive number of viruses, for example on the surface of the respiratory tract, also enables effective horizontal transmission in the population. MERS-CoV is also able

to use the basolateral membrane to release its descendants, which provides access to tissues and blood, more easily resulting in a systemic infection (33).

2.1.6 Immune response

The innate immune system provides the first line of defense against viral infection. In coronaviruses, the mechanisms of innate immune responses have been most comprehensively studied for mouse hepatitis virus (MHV) and SARS-CoV, but little is known about the innate immune responses of commonly circulating human coronaviruses. It has been proposed that endoplasmic reticulum stress and the unfolded protein response – induced mainly by the coronavirus S protein – has a significant role in coronavirus-host interactions and innate immune response (44). It has been shown that MHV infection induces the rapid production of proinflammatory cytokines like interferon (IFN) -α and -β, chemokines, matrix metalloproteinases (MMPs), and the tissue inhibitors of MMPs. These soluble components further activate the cellular part of the innate immunity, such as natural killer (NK)-cells, neutrophils, and monocyte-macrophages (45). The cytokine profiles of SARS-CoV patients have indicated activation of Th1 (T helper 1) cell-mediated immunity and a hyperinnate immune response that leads to the accumulation of alveolar macrophages and neutrophils (46). The higher disease severity of SARS patients has been associated with high levels of certain cytokines, such as IFN-γ, interleukin (IL) 1β, IL-6, and IL-8 (46). Failure in the regulation of IFN response, leading to difficulties in switching from innate immunity to adaptive immunity, has preceded the poor outcome in SARS patients (47). SARS-CoV has been found to distract the immune system and eight of its proteins antagonize IFN response (48). Of the commonly circulating HCoVs, HCoV-HKU1 has been demonstrated to induce type III interferon and proinflammatory chemokine response in infected human alveolar type II cells (49).

Spike protein S is the main inducer of neutralizing antibodies, and nucleocapsid protein N is another major immunogenic component of coronaviruses (21). Both local and systemic antibody responses have been shown to occur in HCoV infection, but in most cases antibody levels in both nasal surfaces and serum decline rapidly. In volunteer studies, reinfection by the same HCoV-229E strain was possible after one year, even though the time of viral shedding was shorter and the symptoms milder compared to the primary infection (50). The overall prevalence of HCoV IgA

antibodies in nasal secretions is lower than the prevalence of serum IgG antibodies, at least in the older population (51). In the majority of SARS patients, specific IgG antibodies and peripheral memory B-cell response vanished in six years, but memory T-cell responses remained active for some of the patients (52).

Whether infection by one HCoV could provide protection from infection by another type has been discussed, and it seems that a previous HCoV-OC43 and -NL63 infection can provide some protection from a subsequent HCoV-HKU1 and -229E infection, respectively, but not vice versa (53). Overall, serologic and neutralization responses against the S protein are mainly strain-specific, and serologic responses against the N protein are cross-reactive only within viruses of the same subgroup (54).

Maternal antibodies for at least HCoV-NL63 and -229E have been found in all newborns but vanish within three months (55).

2.1.7 Diagnostic methods

In a clinical setting, specific laboratory diagnostics are seldom needed for commonly circulating human coronaviruses because they most often cause mild, self-limiting infections. However, the recent development of multiplex respiratory virus PCR tests, which detect several respiratory viruses in a respiratory sample in the same test, have made coronavirus diagnostics easily accessible to many clinical laboratories (56,57). On the other hand, SARS-CoV and MERS-CoV need rapid specific diagnostics because they can cause a severe public health threat and isolation of the positive patients and their contacts is required. PCR, antigen detection methods, and serology form the most accurate diagnostics for SARS-CoV and MERS-CoV infections (58-61).

Several methods have been used in research laboratories to detect and study HCoVs. Early studies used virus culture techniques and EM in addition to serological methods (62-65). In recent years, the development and increasing use of molecular techniques, with PCR at the front line, has improved the sensitivity and specificity of coronavirus detection and left the time-consuming and sometimes difficult culture techniques used less. The replicase gene and N gene regions are commonly used as targets of PCR primers (66-69). Recently, serological methods have also been updated to cover the new members of HCoV family. Nucleocapsid protein N or

parts of it have found to be efficient antigens in enzyme-linked immunosorbent assays (ELISAs) to distinguish the antibodies of separate HCoVs (51,53,55).

2.1.8 Disease spectrum and clinical picture

The disease spectrum of different HCoVs varies from common colds to life-threatening systemic infections. The commonly circulating HCoVs OC43, 229E, NL63, and HKU1 mainly cause mild upper respiratory tract infections, but they are also capable of causing infections – such as bronchiolitis and pneumonia – that require hospital care, especially in children and immunocompromised persons (53,68-72). It has been estimated that 4.8% of lower respiratory tract infections and 3.0% of upper respiratory tract infections of children are caused by HCoVs (68). However, commonly circulating HCoVs have also been detected in asymptomatic children, suggesting the shedding of the virus after symptomatic infections or the possibility that some of the infections really are asymptomatic (73). In one study, NL63 has been associated with Kawasaki disease (74), but in other studies this association has been disproven (75,76).

The clinical picture of SARS- and MERS-CoVs differs substantially from the commonly circulating HCoVs. SARS-CoV causes severe lower respiratory tract infections, resulting in progressive respiratory failure for some patients (77,78). Mortality in SARS-CoV infections was around 10% (79). MERS-CoV can also cause serious pulmonary infection leading to respiratory failure, accompanied by kidney injury and liver dysfunction in a substantial proportion of patients (80,81). The case-fatality rate in MERS-CoV infections is approximately 36% according to the WHO update in June 2015 (http://www.who.int/mediacentre/factsheets/mers-cov/en/). However, both SARS- and MERS-CoVs can also cause asymptomatic and mild infections (81,82).

2.1.8.1 Human coronavirus OC43

HCoV-OC43 was originally isolated in 1967 from a patient with a respiratory tract infection (94). Since then, HCoV-OC43 has been recognized as a respiratory pathogen in both children and adults (95-98). HCoV-OC43 has been the most prevalent HCoV in children (53,68), and the majority of children seroconvert to it before the age of two years (53). The clinical picture in children varies from

infections of the upper respiratory tract to lower respiratory tract infections requiring hospital care, but the former seems to be the more common presentation of the HCoV-OC43 infection (98,99). Detection rates of HCoV-OC43 in hospitalized children have varied from 1.5% to 5.8% depending on the inclusion criteria of the study, study year, and the age distribution of the studied children (53,99,100). The seasonality of HCoV-OC43 infections varies in different geographic locations (66,92,98), but in Europe the peak incidence is in the winter months (101). The circulation of HCoV-OC43 seems to be biennial, although sporadic cases can occur outside the "epidemic" year (101).

2.1.8.2 Human coronavirus 229E

The isolation of HCoV-229E from the respiratory specimens of students with upper respiratory illness was published in 1966 (64). Volunteer studies confirmed that this new virus caused common colds. The incubation period of the virus varied between two to four days and the mean duration of the clinical illness was one week. The extensive use of handkerchiefs by volunteers suggested that nasal discharge is one of the main symptoms of HCoV-229E infection (102). HCoV-229E causes respiratory infections in both children and adults (53,92,96,103), and the clinical picture is usually mild, but the infection may be severe enough to cause hospitalization in children and immunocompromised patients (53,72). However, like other HCoVs, HCoV-229E can also be detected in asymptomatic children (73). Detection rates in children in hospital-based studies have varied between 0.3% and 1.0% (73,92,100). Seroconversion to HCoV-229E occurs in the majority of children before the age of 3.5 years (55).

2.1.8.3 SARS coronavirus

Increasing numbers of severe atypical pneumonia cases arose in China at the end of 2002 and soon patients with the clinical entity now known as SARS were found in North America and Europe in addition to Asia (78,104,105). Within a few months of the onset of the outbreak, the causative agent of SARS was confirmed to be a novel coronavirus (106-108). The natural reservoir of the virus was bats but animals commonly traded in the wild animal markets of China, such as Himalayan palm civets and raccoon dogs, served as a transmission link to humans (109,110).

Common symptoms of SARS-CoV infection included fever, chills, myalgia, and cough, with air-space consolidations in thorax imaging (106,111). Diarrhea was also commonly observed in SARS patients (77,89). Death usually resulted from progressive respiratory failure; histologic analysis of the lungs showing diffuse alveolar damage (78,104,111). The transmission of SARS-CoV mainly occurred via large respiratory droplets during close personal contact (112), but also indirect transmission may be possible because SARS-CoV retains its stability relatively well in the environment (113,114).

2.1.8.4 Human coronavirus NL63

HCoV-NL63 was first isolated from a child with bronchiolitis in 2004 (15). Another group also independently identified this same coronavirus in a child with pneumonia. Interestingly, in this case the positive samples were collected and frozen in 1988, so the virus has been circulating in human population for some time (115). Following studies revealed that this new virus was present in 1.3-3% of children hospitalized for respiratory tract infection around the world (100,116-119), and it was specifically associated with croup (116,119,120). However, even though young children often need a visit to physician during an HCoV-NL63 infection, hospitalizations due to the infections are not very common, suggesting an overall mild clinical picture (121). Both children and adults have been found to be infected by HCoV-NL63 (71,122). HCoV-NL63 has also been found in asymptomatic control cases, which may be a consequence of the shedding of the virus after acute infection (73). As in the case of HCoV-229E, most children seroconvert to HCoV-NL63 before the age of 3.5 years (55). Regarding the substantially low stability of HCoV-NL63 in a dried state on surfaces, the infection route of the virus is probably direct person-to-person transmission, for example via respiratory droplets (123).

2.1.8.5 Human coronavirus HKU1

The fifth HCoV, HCoV-HKU1 was identified in 2005. The first positive sample was a nasopharyngeal aspirate from a 71-year-old man with pneumonia (16). After the primary detection, HCoV-HKU1 has been found in both children and adults (93,103,124). The detection rates in samples of children with respiratory tract infection has varied between 1% and 3.2% depending on the study design (53,69,125,126).

2.1.8.6 MERS human coronavirus

The most recently recognized coronavirus in humans, MERS-CoV (Middle East respiratory syndrome coronavirus), was isolated from a 60-year-old man with fatal pneumonia in Saudi Arabia in 2012 (127). In addition to Saudi Arabia, sporadic cases of MERS-CoV infection were recognized in European countries too, but the background information of the positive cases included a preceding journey in the Middle East region (128,129). The clinical picture resembled that of SARS cases; fever, cough, and dyspnea being the most typical symptoms in the disease onset (80,81). The MERS-CoV genome closely resembles genomes of certain bat coronaviruses (22,128), but dromedary camels are suggested as a main reservoir of the virus (130). Even though the original transmission route of MERS-CoV is still under research, person-to-person transmission has been shown to occur (131).

2.1.9 Human coronaviruses and gastrointestinal infections

Discussion of the existence of human enteric coronaviruses began in the 1970s after the discovery of coronavirus-like particles in stool samples using EM. The association of these proposed enteric coronaviruses and gastroenteritis was not clear because the findings were common in both AGE patients and healthy controls. These enteric coronavirus types were also difficult to cultivate and the meaning of the EM findings remained uncertain (63). In the 1980s, enteric coronaviruses were associated with neonatal necrotizing enterocolitis (NEC), but again attempts to isolate the virus were unsuccessful and findings were based on the morphologic appearance of the virus in stools (83). The cause of NEC has not yet been resolved, but it seems that composition of the intestinal microbiota is a more important factor than a separate pathogen like coronavirus (84).

Since the 1980s the role of HCoVs in gastrointestinal infections was discussed occasionally (85-87), and the successful growth of enteric coronaviruses in cell cultures was also reported (88). However, the role of enteric coronaviruses remained inconclusive.

In 2003 during the SARS-CoV outbreak, it was observed that diarrhea was a common symptom in SARS patients (77,89). In addition, RNA of SARS-CoV was detected in the stool samples of patients, and EM examination of intestinal biopsy samples showed the presence of active viral replication. Despite the active viral

reproduction, there was only minimal damage in the intestinal cells and attempts to isolate SARS-CoV from stool samples were unsuccessful (89). Mucosal lymphoid tissue and intestinal epithelial cells were shown to be infected (90). Higher viral copy numbers in respiratory specimens were associated with the occurrence of diarrhea (91). Whether SARS-CoV directly caused the diarrhea or whether there was some indirect mechanism for the symptoms has not been confirmed.

Molecular studies concerning commonly circulating HCoVs have usually focused on respiratory infections and samples taken from the respiratory tract, but gastrointestinal symptoms have also been observed in some studies (92) and the RNA of HCoV-HKU1 was detected in stool samples in one study (93).

2.2 Human bocaviruses

2.2.1 Classification

Human bocaviruses belong to the genus *Bocavirus* in the subfamily *Parvovirinae* of the family *Parvoviridae*. When the first human bocavirus, HBoV1, was discovered, it was found to be related to bovine parvovirus (BPV) and minute virus of canines (MVC), the original members of the genus *Bocavirus*, hence the name human bocavirus. This relationship was defined by the similarity of the sequence and organization of the genome. The amino acid identity between HBoV1 and these animal viruses was approximately 40% similar in the major ORFs (17). Later, three new human bocaviruses (HBoV2-4) were identified (18-20). Even though HBoVs were discovered during the last ten years, the viruses have circulated for a longer time; HBoV1-3 were detected in the retrospective screening of samples collected in the 1980s (132).

Other viruses in the *Parvovirinae* subfamily that are found in human specimens include parvovirus B19, adeno-associated virus, parvoviruses 4 and 5 (parvovirus 4 genotype 2), and bufavirus (133-136). So far, only parvovirus B19 has been proven to cause disease in humans (133).

The taxonomy of viruses in the family *Parvoviridae* has recently undergone significant changes. The changes for *Bocaviruses* in the International Committee on Taxonomy of Viruses study group proposal includes new genus name, *Bocaparvoviruses*. New species name, *Primate bocaparvovirus 1*, was created to include HBoV1, HBoV3, and Gorilla bocavirus, and *Primate bocaparvovirus 2* to include

HBoV2 and HBoV4. Viruses that encode NS1 protein that show over 85% amino acid identity belong to same species. (137)

2.2.2 Structure

Viruses belonging to the family *Parvoviridae* are small, around 18-26 nm in diameter, nonenveloped, and have an icosahedral capsid, which consists of 60 capsid proteins. The capsid is structurally fairly stable and tolerates a pH between 3 and 9 and a temperature of 56°C for one hour. The genome is a linear negative- or positive-sense single-stranded DNA with hairpin structures at each end in most of the viruses (133). In the HBoV capsids, most of the DNA is negative-stranded (138).

In EM, HBoV particles present hexagonal structures typical for members of the *Parvoviridae* (139). The detailed capsid topology of HBoVs is a mixture of the features of the other members in the *Parvoviridae* family and is the most similar to the capsid structures of parvovirus B19. The VP2 protein is the main building block of the HBoV capsid (140).

2.2.3 Genome and proteins

The HBoV genome is around 5,000 bases in length and contains three ORFs. The first ORF encodes a nonstructural protein, NS1, the second ORF encodes another nonstructural protein, NP1, and the third ORF encodes VP1 and VP2, the structural capsid proteins (Figure 3). The middle ORF encoding the NP1 is unique to viruses in the genus *Bocavirus* (17).

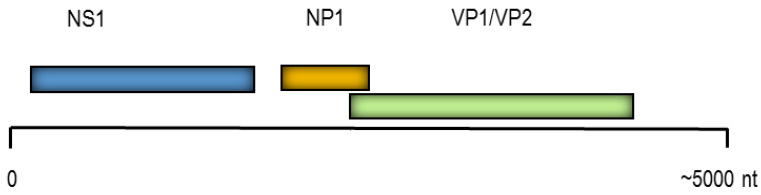


Figure 3. Schematic representation of the genome organization of human bocaviruses. Boxes represent different genes. (Modified from 141)

The NS1 protein includes conserved motifs that are associated with replication and helicase and ATPase functions (20). In addition to the large NS1 protein, the NS1 gene is also expressed in smaller sizes (142). A recent study confirmed the existence of three additional small NS proteins (NS2, NS3, and NS4) and at least NS2 was essential for HBoV1 replication in an HAE culture (143). The function of the NP1 protein is obscure, but there is some evidence that this protein could induce apoptotic cell death in infected cells and also disturb IFN response (144,145). Both NS1 and NP1 are localized in the cell nucleus during infection, so both probably play a role in DNA replication (142). VP1 and VP2 have identical amino acid sequences except for the additional 129 amino acids in the amino-terminal region of VP1, which is called the VP1 unique region (146). This region probably plays an important role in the pathogenesis of HBoV1 in the respiratory tract (147).

Recombination events have shown to be common in HBoVs (18,20,148-151) and there are suggestions that HBoV3 formed from a recombination event of HBoV1 and HBoV2, and HBoV4 formed from recombination of HBoV2 and HBoV3 (18,20). In another study, HBoV3 was proposed to be a result of recombination between HBoV1 and HBoV4 (148). There is also evidence of intra-genotype recombination among HBoV2 variants (20,152,153). Overall, HBoV2-4 strains are genetically more dispersed compared to HBoV1 strains, which are genetically closely related and show only minor sequence variation (20,153-156). It has been proposed that HBoV1 recently evolved from an enteric bocavirus ancestor and got its respiratory tropism through mutation or recombination (20).

2.2.4 Replication cycle

2.2.4.1 Virus entry

Generally, members of the *Parvoviridae* family use receptor-mediated endocytosis for cell entry. Following endocytosis, virus particles are released into the cytoplasm and transported to the nucleus for replication (133). However, specific mechanisms and receptors for the cell entry of HBoVs remain thus far unsolved.

2.2.4.2 Replication and transcription

Because most viruses of the *Parvoviridae* family lack the DNA polymerase enzyme, they need the DNA replication machinery of the infected cell for successful reproduction (133). Parvoviruses are in general assumed to use the so-called rolling-hairpin model in their replication, but this model seems unlikely in HBoVs because their terminal sequences do not fit the rolling-hairpin model (157,158). In addition, head-to-head and tail-to-tail replicative intermediates, typical of the rolling-hairpin model, have not been detected for HBoVs (159).

The transcriptional patterns of different parvoviruses vary significantly (133). The transcription profile of HBoV1 has been shown to resemble that of BPV and MVC. All types of HBoV1 mRNAs are transcribed from a single promoter and then processed through alternative splicing and polyadenylation (142).

2.2.4.3 Assembly and release of virions

Generally, after translation in the cytoplasm, parvovirus capsid proteins are transported to the nucleus for assembly. However, this process is not fully understood (133). Release of the mature HBoV virions can occur from both apical and basolateral sides of the infected cell (160,161).

2.2.5 Pathogenesis

HBoVs are difficult to cultivate in conventional cell lines and this, in addition to lack of suitable animal model, has hindered the study of the pathogenetic mechanisms of HBoV infection. However, differentiated human airway epithelial (HAE) cells have been successfully used in an in vitro model for HBoV1 infection, and a reverse genetics system using a plasmid clone of HBoV1 and human embryonic kidney 293 cells has been generated for the production of viruses (141,160). In addition, two commercially available HAE cultures have recently been shown to support the growth of HBoV1 (162).

In the HAE cell model, HBoV1 infection caused disruption of the tight junction barrier, epithelial cell hypertrophy, and the loss of cilia, which are signs of respiratory tract damage (160,161). The unique region of the VP1 protein has been shown to

cause disruption of the tight junctions in human airway epithelial A549 cells and is probably an important factor in HBoV1 pathogenicity (147). It has been suggested that damage in the epithelial lining of the respiratory tract during an HBoV1 infection may expose the host to secondary infections by other microbes and increase the permeability of the epithelia to allergens. In addition, a lack of cilia may predispose to bronchiolitis (160). HBoV1 is probably able to reproduce in non-ciliated cells as well, because HBoV1 mRNA and high levels of DNA have been detected in palatine tonsil tissue (163).

HAE cell models have also shown that HBoV1 can enter the cell from both apical and basolateral surfaces, but infection through the apical surface is more efficient. Viruses can also be released from both surfaces (161). Viremia in HBoV1 infection may be a consequence of basolateral release or the leakage of viruses from the surface of the epithelium because of damage in the cell junctions (160,161).

The pathogenetic mechanisms of other HBoVs are yet to be characterized.

2.2.6 Immune response

The NP1 protein of HBoV1 has been found to suppress INF-β production by interfering the interferon regulatory factor (IRF)-3-signaling pathway. This interference is probably a consequence of the direct binding of NP1 to IRF-3 in the nucleus, which blocks the association of IRF-3 and the INF-β promoter (145). Conversely, the VP2 protein of HBoV1 has been shown to upregulate the expression of INF-β and it has been proposed that increased INF-β levels may facilitate the formation of a latent HBoV1 infection. Based on the findings of these two opposite mechanisms, it has been suggested that NP1 and VP2 function at different stages of the virus life cycle: NP1 is an early gene and VP2 is expressed in the late stage of infection (164). Interestingly, simultaneous HBoV1 infection has been found to modulate the immunological responses towards rhinovirus in wheezing children (165).

HBoVs are capable of eliciting a systemic B-cell response, but this response seems to be weaker in HBoV2-4 compared to HBoV1 (166). HBoV1 antibodies are more stable compared to others, but there are significant differences between individuals (167). The main target of the antibodies is the HBoV capsid protein VP2, whereas the unique region of VP1 has been shown to be less immunoreactive (168). HBoV antibodies are cross-reactive (166,169) and it has been proposed that the original antigenic sin (OAS) phenomenon is involved in the poor antibody response in

heterologous secondary HBoV infections (167). The question of whether HBoV antibodies are cross-protective between genotypes requires further studies (167).

Cell-mediated immune responses have been detected towards HBoV1 VLPs. These T-cell responses weaken with increasing age (170).

2.2.7 Diagnostic methods

PCR methods have been used in HBoV diagnostics widely, but frequent HBoV detections in asymptomatic persons have made the sole use of this method problematic. However, a high DNA load (>10,000 copies/ml) in the nasopharynx correlate with serological markers for acute infection (168,171,172), and viremia is generally a good indicator of acute HBoV1 infection (173) even though HBoV DNA may appear at low levels in the blood of asymptomatic subjects (174,175). The detection of spliced mRNA has also been shown to be a usable marker for acute, active HBoV1 infection and to correlate with a high DNA concentration in the nasopharynx and viremia (176). Mostly, the NS1 gene region has been used as a target for PCR primers (155,177,178), but also the VP1 and NP1 gene regions have been employed (20,179,180).

Serological methods have successfully used the VP1 protein, VP2 protein, and virus-like particles (VLPs) as antigens (166,168-170,181,182). Conserved B-cell epitopes of HBoVs have also been used in diagnostic tools and have shown to be comparable to VLP-based methods (183). Western blot (172), immunofluorescence assay (181), and EIAs have been employed (166,170,172,182). IgG-avidity assays have also been developed for HBoV1 (184). Cross-reactivity of antibodies between different HBoVs has complicated the serodiagnostics of HBoVs (166,169), and competition assays are needed for correct results (166,182). In addition, a lack of an IgG increase in heterologous secondary infections, most probably due the OAS phenomenon, hampers the use of serological methods (167,185).

2.2.8 Disease spectrum and clinical picture

2.2.8.1 Human bocavirus 1

HBoV1 was originally detected in nasopharyngeal samples and suggested to be a respiratory pathogen in children (17). Since its discovery, studies worldwide have reported HBoV1 in 4.4-25% of children with a broad variety of respiratory symptoms (154,171,176,186-198). Specific conditions associated with HBoV1 include wheezing and pneumonia (173,191,199), even though a large meta-analysis of the viral cause of acute lower respiratory tract infections did not find a causal linkage to HBoV1 in these conditions (200). Because co-detections with other respiratory viruses have been common (34.6-90% of the cases) (154,172,186,194,196,201-204), and since HBoV1 has also been found in asymptomatic children (197,202,205-207), the role of HBoV1 as a respiratory pathogen has been doubted. However, detection of HBoV1 alone, viremia, and a high viral load in respiratory samples are associated with respiratory infections in children (202). A recently published longitudinal study also showed a connection between HBoV1 primary infection and mild respiratory symptoms, adding to the evidence that HBoV1 is a cause of respiratory infections (208). In sporadic cases, HBoV1 has even been associated with life-threatening respiratory infections in children (209-212). Children under two years of age are more commonly infected than older children (155,172). Detection of HBoV1 in adults is not common (188,194,206,213), but a severe HBoV1 infection requiring intensive care has been reported in the case of a 74-year-old man (214).

Because diarrhea has been among symptoms in children with respiratory infection and HBoV1-positive respiratory samples, it has been suggested that HBoV1 may have a role in gastroenteritis. One study showed that diarrhea was more common in cases with a very high HBoV1 viral load ($>10^8$ copies/ml) in the respiratory samples compared with those with a lower viral load (215). In another study, when compared to a lower viral load, no association was found between a high viral load in the respiratory tract and diarrhea. In this study, $>10^6$ copies/ml were used as a limit for a high viral load (178). The median viral load of HBoV1 in nasopharyngeal aspirate samples has been higher in cases with HBoV1-positive stool samples compared to cases with negative stool samples, raising suggestions that HBoV1 DNA in stools may be swallowed from the respiratory tract (190). The median HBoV1 load in stool samples has been lower compared to the load in respiratory samples (216). Overall, HBoV1 has been found in 0.8-10% of the stool

samples of children with AGE, co-detections with other viruses being found in 36-100% of the cases (213,217-225). Despite frequent detection in stools, four case-control studies did not find an association between HBoV1 and gastroenteritis (18,224,226,227). In addition, it has been shown that co-infection with HBoV1 does not worsen the clinical picture of rotavirus gastroenteritis (220). Thus, current results do not support the role of HBoV1 as a cause of gastroenteritis (18,220,224,226,227).

HBoV1 DNA has also been detected in the cerebrospinal fluid (CSF) of children with encephalitis without other potential pathogens (228), and a case of invasive HBoV1 infection with hepatitis and skin manifestations has been reported in a child with T-cell deficiency (229). Because animal bocaviruses BPV and MVC are known to infect fetuses and to cause adverse pregnancy outcomes, the association of HBoV1 with these conditions has also been studied, but no connection was found (230).

HBoV1 DNA has been detected at low levels in respiratory samples and urine 31 days after acute infection (172), and even after six months in nasal swabs obtained monthly from asymptomatic children (205). In a recent longitudinal study, HBoV1 was detected in dried oral fluid samples after more than one month in 64% of children after primary infection, and in some children even after more than a year (208). HBoV1 was detected in the stool samples of a healthy child for 51 days (231). HBoV1 DNA can also be found in the sera of children without symptoms of any infection (232). Frequent detection of HBoV1 from asymptomatic persons indicates that prolonged shedding after acute infection – or asymptomatic carriership – is common (171). Prolonged replication and persistence is common for several animal parvoviruses and also for parvovirus B19, which infects humans and occasionally causes chronic persistent infection (133).

In a large follow-up study with longitudinally collected serum samples, 80% of the children became HBoV1 seropositive before the age of six years, the mean age for seroconversion being 1.9 years. Maternal antibodies were found in 51% of children (167). In a larger scale, the HBoV1 seroprevalence was 50% in healthy children (0-14 years) and 66.9% in healthy adults (182). Based on serology, HBoV1 is the most prevalent HBoV (166,167,182).

HBoV1-positive cases have been found around the year (178,187,215,220,233), with the highest detection rates mainly observed in winter and fall (187,213,220), but peak incidence has also been detected in spring and summer (227,233). Variations in average detection rates between the seasons have been observed (215).

2.2.8.2 Human bocavirus 2

HBoV2 was discovered in 2009 in stool samples screened using viral metagenomics (19). Since its discovery, several studies have detected HBoV2 DNA in 1.4-24.6% of children with AGE, and co-detections have been found in 53.8-100% of cases (18,179,221-224,234). In a large, age-matched, case-control study, HBoV2 was detected at similar rates in cases and controls in children under five years of age. In the older age groups of the same study, HBoV2 was detected more often in the feces of the cases compared to the controls, but the exact numbers were small (227). In an Australian case-control study, an association between HBoV2 and AGE in children was found (18). A third case-control study conducted in China also found a statistical association between HBoV2 and AGE in children, but further analyses showed that HBoV2 was less prevalent in samples without any other gastroenteritis viruses compared to cases where a known gastroenteritis virus was detected. In addition, co-infection with HBoV2 did not exacerbate the clinical severity of AGE cases, and overall, the HBoV2 virus loads were low and did not differ between the cases and controls. The authors concluded that HBoV2 was not a cause of AGE in their study (224).

HBoV2 has been detected in 0.6-4.3% of respiratory samples of children with ARTI, usually together with other viruses (233,235,236). HBoV2 has also been detected in stool samples of children with ARTI (237).

HBoV2 DNA has also been detected in the CSF of children with encephalitis, and bocavirus-like particles have been seen in the CSF by EM, indicating that HBoV2 is capable of crossing the blood-brain barrier. However, further investigation is required to determine of the exact role of HBoVs in encephalitis (228). There is also a case report of subacute myocarditis associated with HBoV2 that led to the death of a 13-month-old child (238).

In a large follow-up study of children, an average age for HBoV2 seroconversion was 1.6 years, and 48% became seropositive before the age of six years (167). HBoV2 seroprevalence in healthy children (0-14 years) was 36.9%, whereas 49.3% of healthy adults had HBoV2 antibodies when using a competition ELISA assay (182). HBoV2 is the second most prevalent HBoV (167,182).

HBoV2-positive cases have been found throughout the year without a clear seasonal variation (179).

2.2.8.3 Human bocavirus 3

HBoV3 was detected for the first time while screening stool samples of children with AGE for HBoV2 (18). HBoV3 DNA has been found in 0.5-2.7% of stool samples of children with AGE (18,222-224), the co-detection rate being 50-100% (222-224). Overall, HBoV3 detection rates in children with AGE have been lower than those of HBoV2 (222,224,239). According to three case-control studies, there is no association between HBoV3 and AGE (18,224,227). HBoV3-positive cases have been found mostly in young children (227).

Detection of HBoV3 in respiratory samples has been rare, but in a study conducted in Japan, 0.4% of respiratory samples of children with ARTI were positive for HBoV3 (233).

HBoV3 antibodies have been found in 28.7% of healthy children (0-14 years) and in 38.7% of healthy adults when a competition ELISA assay has been employed. HBoV3 is the third most prevalent HBoV according to seroprevalence (182). Ten percent of children become HBoV3 seropositive before the age of six years, the average age at seroconversion being 1.7 years (167).

2.2.8.4 Human bocavirus 4

HBoV4 was discovered in 2010 in stool samples (20) and since then it has been detected occasionally in the stools of children with AGE, detection rates varying 0-0.5% (222-224). In addition to stool samples, one study detected HBoV4 in 0.6% of respiratory samples of children with ARTI (233).

HBoV4 seroprevalence has been reported as 0.8% in healthy children (0-14 years) and 1.4% in healthy adults when measured by a competition ELISA assay; HBoV4 is thus the least common HBoV in the community (182).

3 Aims of the Study

Noroviruses, rotaviruses and adenoviruses cover the majority of the AGE cases in children in Finland. In occasional cases sapoviruses and astroviruses are causes of the AGE symptoms. However, despite the comprehensive diagnostic methods, there are still cases of AGE in children without a known causative pathogen. The general aims of this study were to find pathogens that would fill this "diagnostic gap". The specific objectives of this dissertation were the following:

1. To determine if commonly circulating human coronaviruses can be detected in stool samples of children with acute gastroenteritis, and to evaluate the significance of human coronavirus findings in stools as a possible causative agent of acute gastroenteritis in children

2. To determine the occurrence of human bocavirus types 1-4 in stools and the respiratory tract of children hospitalized for acute gastroenteritis, and to evaluate the significance of human bocavirus findings in stools as a possible causative agent of acute gastroenteritis in children

4 Materials and Methods

4.1 EPI I study, September 2006 – August 2008 (I, III)

The EPI I material was collected from Tampere and Kuopio University hospitals between September 2006 and August 2008. The study was approved by the Ethics Committee of Pirkanmaa Hospital District. Children under 15 years of age with AGE who visited the pediatric emergency room (ER), were admitted to the hospital ward, or came down with AGE during hospitalization were eligible for the study. The diagnosis of AGE was made by a physician in the pediatric department. Three groups of control children without symptoms of gastroenteritis were also enrolled. Informed consent was obtained from the parents or legal guardians of all the participating children. A stool specimen was collected from all children. In addition, parents were interviewed about their child's symptoms preceding the hospital visit, and information about the hospital visit was gathered from the medical records. In addition, a questionnaire about the duration of the symptoms after the discharge was requested to be completed by the parent/guardian and returned after the child's recovery. If a child had more than one hospital visit during the study period, the visits were considered as two separate episodes when there were at least seven asymptomatic days between the admissions. The material was originally collected for epidemiological study of rotavirus and norovirus by Sirpa Räsänen (6,240).

For this dissertation, 990 stool samples were available. Most of the samples, approximately 92%, were collected from Tampere University Hospital. A total of 878 stool specimens were from children with AGE, 43 from children with indeterminate fever and vomiting, 33 from children with ARTI, and 36 from healthy children. The characteristics of the study groups are introduced in Table 1.

In addition to HCoVs and HBoVs, rotaviruses and caliciviruses, including norovirus genogroups I and II and sapoviruses, were studied from all stool samples (6,240). In addition, some of the specimens were studied for adenovirus (101 specimens before Study I and 724 specimens before Study III, unpublished data).

Table 1. Characteristics of the study groups for the EPI I material.

	N	Male %	Age (median)	Number of children in the different age groups			
				<6 months	6-24 months	2-5 years	>5 years
AGE group	878	55.7	18 months	80 (9.1%)	473 (53.9%)	230 (26.2%)	95 (10.8%)
Control groups	112	62.5	16 months	19 (17.0%)	49 (43.8%)	33 (29.5%)	11 (9.8%)
1.	43	60.5	17 months	10 (23.3%)	14 (32.6%)	13 (30.2%)	6 (14.0%)
2.	33	63.5	14 months	4 (12.1%)	18 (54.5%)	10 (30.3%)	1 (3.0%)
3.	36	63.9	19 months	5 (13.9%)	17 (47.2%)	10 (27.8%)	4(11.1%)

1. indeterminate fever and vomiting

2. ARTI

3. healthy

4.2 EPI II study, September 2009 – August 2011 (II, IV)

The EPI II study was conducted at Tampere University Hospital's Department of Pediatrics between September 2009 and August 2011. The study was approved by the Ethics Committee of Pirkanmaa Hospital District. Children under 16 years of age with AGE who were visiting the pediatric ER, were admitted as inpatients, or had AGE during a stay in the hospital were eligible for the study. Of the children with ARTI, only those admitted to the hospital ward were eligible. Informed consent was obtained from the parents or legal guardians of all children enrolled. If a child visited the hospital more than once during the study period, the admissions were considered to represent separate episodes when the child had been healthy for at least 2 weeks between the admissions. Stool samples and nasal swab samples were collected from all children if possible. Blood samples were collected if blood was taken for diagnostic or treatment-related reasons.

In 955 cases, both stool and nasal swab samples were available, and these cases were studied for this dissertation. In addition, 288 acute phase serum samples were available from these 955 cases. For the analysis of the results, the patients were divided into three groups: the AGE group included 172 children with symptoms of gastrointestinal infection only, the ARTI group included 545 children with symptoms of respiratory tract infection only, and the AGE/ARTI group included

238 children with different symptom combinations of both AGE and ARTI. The characteristics of the study groups are shown in Table 2.

In addition to HCoVs and HBoVs, all stool samples were examined for rotaviruses, and the stool specimens of the children in the AGE and AGE/ARTI groups were examined for caliciviruses (including noroviruses and sapoviruses) (7,241). Those stool specimens that were positive for HCoVs or HBoVs were additionally tested for adenoviruses and astroviruses.

Table 2. The characteristics of the study groups for the EPI II material.

	N	Male	Age	Number of children in the different age groups			
		%	(median)	<6 months	6-24 months	2-5 years	>5 years
AGE[a] group	172	61.6	20 months	31 (18.0%)	63 (36.6%)	48 (27.9%)	30 (17.4%)
ARTI[b] group	545	62.9	13 months	156 (28.6%)	275 (50.5%)	93 (17.1%)	21 (3.9%)
AGE/ARTI[c] group	238	61.3	13 months	37 (15.5%)	143 (60.1%)	37 (15.5%)	21 (8.8%)

[a]acute gastroenteritis

[b]acute respiratory tract infection

[c]symptoms of both AGE and ARTI

4.3 Sample collection and storage

Study nurses or nurses working in the pediatric department collected stool samples in empty sample tubes mainly from diapers. If a child did not produce stools during the hospital stay, in some cases study nurses collected a sample from the child's home after the discharge (EPI I and EPI II) or equipment was provided to the parent/guardian for taking a sample at home and sending it to the research laboratory (EPI II).

Nasal swab samples were collected using flocked swab sticks in UTM-RT Mini tubes (Copan Italia, Brescia, Italy) to support viral survival. Samples were collected by study nurses or nurses working at the pediatric department by swabbing the patient's nostril to a depth of 2-3 centimeters. Nasal swab samples were collected only in the EPI II study.

If stool or nasal swab specimens were not delivered to our research laboratory immediately after they were taken, they were kept at +4 ºC until delivery was possible. In the research laboratory, the specimens were stored at -20 ºC until studied.

Whole blood samples and serum samples in EPI II study were collected by laboratory nurses working in the laboratory of the Tampere University Hospital (Fimlab laboratories, former Laboratoriokeskus) or a sample was taken through intravenous cannula by study nurses or nurses working at the pediatric department. Blood samples were processed and the serum divided in the laboratory of the hospital; it was stored at -70 ºC until delivered to research laboratory.

4.4 Laboratory methods

4.4.1 Preparation of samples for extraction

For nucleic acid extraction, stool specimens were diluted in phosphate-buffered saline (PBS) to prepare 10% suspensions. Nasal swab samples in UTM-RT tubes were mixed and centrifuged, and transport medium was directly used for extraction. Serum and whole blood samples were used as they were for extraction.

4.4.2 Extraction of viral RNA and DNA

Viral nucleic acid was extracted from stool suspensions and the nasal swab transport medium using a QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The kit uses a spin column technique with a silica-gel-based membrane to extract nucleic acids. This kit was used for both coronaviruses and bocaviruses after also being tested for suitability for DNA extraction. For the serum samples, either a QIAamp DNA mini kit (QIAGEN) or a QIAamp Viral RNA Mini Kit was used. The extracted RNA or DNA were divided into aliquots of 12 µl and stored at -70 ºC until used for PCR. Freezing-thawing cycles were done only once or twice before amplification of nucleic acids.

4.4.3 RT-PCR method for the detection of human coronaviruses (I, II)

4.4.3.1 Reverse transcription

Before the actual coronavirus PCR procedure, reverse transcription was done for the extracted samples using random primers as described by Pang et al. 2005 (242), with minor modifications. Briefly described, a master mix containing 1 χ First Strand Buffer (Invitrogen, USA), 5 μM DTT, 200nM dNTP mix, 600 ng random primers, 20 units RNaseOut, and 100 units Superscript II was prepared. From this mixture, 15 μl was combined with 5 μl of the extracted sample and kept at +42 ºC for 60 minutes and at +70 ºC for 15 minutes. The produced cDNA was stored at -20 ºC if not used directly for PCR. All lists included one negative control (aqua sterilisata) for every ten samples and one positive control per list.

4.4.3.2 PCR

A two-step PCR method was used for the detection of HCoV RNA. This method was set up and optimized in our laboratory. Method specificity was tested using positive samples for 31 viruses and four bacteria. All PCR lists included one negative control (aqua sterilisata) for every 10 samples and one positive control per list. HCoV strains TC-adapted OC43 (VR-1558) and 229E (VR-740), purchased from ATCC (USA) and propagated in HCT-8 and MRC-5 cells respectively, were used as positive controls.

Primers HCoV nsp12 fwd and HCoV nsp12 rev (Table 3) were designed to amplify a part of the conserved polymerase gene region universal to all HCoVs and produce a 438 bp amplicon in the first PCR step. In this step, 10 μl of the cDNA was added to 40 μl of the reaction mixture, which contained 1 χ Green GoTaq Flexi Buffer (Promega, USA), 2.5 mM of GoTaq $MgCl_2$ (Promega), 200 μM of each dNTP (Promega), 2.5 U of GoTaq DNA polymerase (Promega), and 0.5 μM of each primer (Sigma-Genosys Ltd., UK). Temperatures and cycles in the PCR program were the following: 2 min at 94 ºC, 35 cycles of amplification (30 sec at 94 ºC, 30 sec at 54 ºC, 1 min at 72 ºC), and a final extension at 72 ºC for 5 min.

The second step of the PCR contained three primer pairs designed to separate three coronavirus groups including, 1B (now known as part of alphacoronaviruses, including HCoV-229E and -NL63), 2A (now betacoronavirus group A including HCoV-OC43 and -HKU1,) and SARS (betacoronavirus group B) (Table 3). Two

microliters of the first PCR product were added to 48 µl of the reaction mixture, which contained 1 χ Green GoTaq Flexi Buffer (Promega), 1.5 mM of GoTaq $MgCl_2$ (Promega), 200 µM of each dNTP (Promega), 2.5 U of GoTaq DNA polymerase (Promega), and 0.5 µM of each primer (Sigma-Genosys Ltd.). The following PCR program was employed: 2 min at 94 ºC, 35 cycles of amplification (30 sec at 94 ºC, 30 sec at 53 ºC, 30 sec at 72 ºC), and a final extension at 72 ºC for 5 min. GeneAmp PCR system 9700 (Applied Biosystems, USA) or Thermal Cycler 2720 (Applied Biosystems) was used to run the PCR programs.

PCR products were visualized using agarose gel electrophoresis.

4.4.4 Qualitative PCR method for the detection of human bocaviruses (III, IV)

HBoV DNA was detected using a two-step PCR method, which was set up and optimized in our laboratory. Method specificity was tested using positive samples for 27 viruses and four bacteria. All PCR lists included one negative control (aqua sterilisata) for every 10 samples and one positive control per list. An HBoV1-positive sample obtained from Karolinska Institutet was used as an original positive control.

In the first PCR, a primer pair was used to amplify a 959-bp amplicon of non-structural protein gene NS1 (Table 3). In the first step, the reaction volume of 50 µl contained 5 µl of the sample DNA, 1 χ Green GoTaq Flexi Buffer (Promega), 1.5 mM of GoTaq $MgCl_2$ (Promega), 200 µM of each dNTP (Promega), 2.5 U of GoTaq DNA polymerase (Promega), and 0.5 µM of each primer (Sigma-Alrich, St Louis, USA). The temperatures and cycles in the PCR program were as follows: 3 min at 94 ºC, 35 cycles of amplification (40 sec at 94 ºC, 30 sec at 64 ºC, 65 sec at 72 ºC), and a final extension at 72 ºC for 5 min.

The second step of the PCR contained two pools of primers, pool Boca (155) and pool HBoV, which produced amplicons of 290 and 199 bp in size, respectively (Table 3). Pool Boca primers detected HBoV1 and pool HBoV primers detected all HBoVs. The reaction volume was 50 µl, containing 2 µl of the first PCR product, 1 χ Green GoTaq Flexi Buffer (Promega, USA), 1.5 mM of GoTaq $MgCl_2$ (Promega), 150 µM of each dNTP (Promega), 2.5 U of GoTaq DNA polymerase (Promega), and 0.5 µM of each primer (Sigma-Alrich). The PCR program was run as follows: 3 min at 94 ºC, 30 cycles of amplification (30 sec at 94 ºC, 30 sec at 55 ºC, 30 sec at 72 ºC), and 5 min at 72 ºC.

GeneAmp PCR system 9700 (Applied Biosystems) or Thermal Cycler 2720 (Applied Biosystems) were used to run the PCR programs.

PCR products were visualized using agarose gel electrophoresis.

Table 3. The oligonucleotide primers for HCoV and HBoV. (IUB, the International Union of Biochemistry, codes in bold: S=G or C, W=A or T, K=G or T, R=A or G, Y=T or C, M=A or C, N=G, A, T, or C)

Method	Primer	Sequence 5'→ 3'	Position	Product
HCoV RT-PCR				
1st PCR	HCoV nsp12 fwd	G**W**TGGGA**Y**TATCC**N**AA**R**TGTGA	14327-14348[a]	
	HCoV nsp12 rev	**YR**TCATCA**SWN**A**R**AATCATCAT	14743-14764[a]	438 bp
2nd PCR	HCoV 1B fwd	GTTGTTTATTC**W**AATGGTGG	14476-14495[a]	
	HCoV 1B rev	**Y**CTATA**R**CAATTATCATA**M**AG	14659-14679[a]	204 bp
	HCoV 2A fwd	**WY**T**R**CGTATTGTTAGTAGTTT**R**GT	15191-15214[b]	
	HCoV 2A rev	CGTATACT**W**A**R**ATCTTCAATCTT	15444-15466[b]	276 bp
	HCoV SARS fwd	TGCTGTAACTTATCACACCGT	15303-15323[c]	
	HCoV SARS rev	CGGACATACTTGTCAGCTATCT	15511-15532[c]	230 bp
HBoV PCR				
1st PCR	HBoV NS1 fwd	GGACGTGGT**S**CGTGGGAAC	1089-1107[d]	
	HBoV NS1 rev	GTCCTGTGAATG**W**GTAGGACAAAGG	2024-2048[d]	960 bp
2nd PCR	HBoV NS1 2nd fwd	CC**W**GTAATTAT**W**TCCACTAACCA	1764-1786[d]	
	HBoV NS1 2nd rev	AGAGTACA**K**TCGTACTCATT**R**AA	1941-1963[d]	200 bp
	Boca NS-1 fwd*	TATGGCCAAGGCAATCGTCCAAG	1545-1567[d]	
	Boca NS-1 rev*	GCCGCGTGAACATGAGAAACAGA	1813-1835[d]	291 bp

* by Sloots et al. 2006 (155)

[a] in the genome of isolate AF304460 Human coronavirus 229E

[b] in the genome of isolate AY391777 Human coronavirus OC43

[c] in the genome of isolate AY274119 SARS coronavirus TOR2

[d] in the genome of isolate GQ200737 HBoV2 KU1

4.4.5 Quantitative PCR method for the detection of human bocaviruses (IV)

HBoV DNA was detected in serum samples using the quantitative PCR method described by Kantola et al. (177). The primers covered a sequence between the left-hand untranslated region and the beginning of the NS1 gene. The hydrolysis probe covered a region fully conserved between HBoVs and was labeled with the reporter dye FAM from its 5' end and blocked with non-fluorescent quencher BHQ1 from its 3' end. Quantitative PCR was done using the Stratagene Mx3005P system (Stratagene, USA). The reaction volume of 25 µl included 1 χ TaqMan Universal Master Mix (Applied Biosystems) with AmpErase uracil-N-glycosylase (UNG), 0.6 µM of each primer, 0.3 µM of the probe, and 2 µl of the template. The PCR program was run as follows: 2 min at 50 ºC , 10 min at 95 ºC, and 40 cycles of amplification (15 sec at 95 ºC, 1 min at 60 ºC).

Laboratory works involving this method were done by collaborators at the University of Helsinki.

4.4.6 Enzyme immune assay method for the detection of human bocavirus antibodies (IV)

HBoV antibodies were studied using the VLP-based EIA method described by Kantola et al. (166). The method is represented in more detail in the doctoral dissertation of Kalle Kantola (243).

In the IgG assays, biotinylated VLPs were attached to streptavidin-coated polystyrene plates, and 100 µl of diluted sample serum (diluted in 1:200 of PBSP, PBS+0.05% polysorbate 20) was added to the micro wells in the plates and incubated for one hour at room temperature. After incubation, the plates were washed three times with PBSP and then incubated with horseradish peroxidase-conjugated rabbit anti-human IgG (DAKO, Denmark) for one hour and then washed four times. After this, a mixture of orthophenylene diamine substrate and H_2O_2 was added, and finally the termination of the reaction was done using H_2O_4. The absorbances were read at 492 nm.

IgM assays were done in the µ-capture format. Plates were first coated with goat anti-human IgM (Cappel/ICN Biomedicals, USA), and after this, diluted serum samples were added and the plates were incubated at room temperature for one hour. After washing, HBoV VLPs were applied and the plates were incubated for 45 min

at 37°C, and washed. Then horseradish peroxidase-conjugated streptavidin (DAKO) was added, incubated, and washed again. Finally, a mixture of o-phenylenediamine dihydrochloride substrate (DAKO) and H_2O_2 was added. The termination of the reaction was done using H_2O_4 and the absorbances were read at 492 nm.

Because of the cross-reactivity of the HBoV antibodies, antibody absorption assays were used to measure genotype-specific HBoV antibodies. These competition assays used a soluble antigen that bound to cross-reactive antibodies and thus only specific antibodies were left to bind the immobilized antigen.

Laboratory works involving this method were done by collaborators at the University of Helsinki.

4.4.7 Sequencing

Positive PCR products (in stool and nasal swab samples) were sequenced. PCR primers (primarily forward primers) were used as sequencing primers, and amplicons were gel-purified with QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. Sequencing was done employing a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, USA) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The prepared sequences were analyzed and processed utilizing the Sequencher 4.9 program (Gene Codes Corporation, USA). The specific virus type was determined by comparing the sequence to the reference strains in GenBank using BLAST (US National Library of Medicine, USA).

4.4.8 Methods for the other viruses studied

Rotaviruses were detected using the RT-PCR method. During the first PCR step, a full-length copy of gene 9 encoding the VP7 protein was amplified; the following nested PCR step included genotype-specific primers detecting commonly circulating genotypes (6,244,245). For samples in the EPI II study, the rotavirus RT-PCR was slightly updated.

Caliciviruses were studied using the RT-PCR method with a primer mixture localizing in the RNA polymerase region A in ORF 1, detecting both noroviruses and sapoviruses. Positive PCR products were sequenced (241). Adenoviruses and astroviruses were detected using ProSpecT EIA kits according to manufacturer's instructions (Oxoid, UK). Adenovirus antigen-positive samples were further studied

by PCR, which separated the enteric adenovirus types from other types (method based on Allard et al. 1992 (246)).

4.5 Statistical methods

Statistical analyses were done utilizing the IBM SPSS Statistics 18 or 20 programs (IBM Corp., USA). Fisher's exact test or the χ^2 test were used as appropriate and p-values below 0.05 were considered statistically significant.

5 Results and discussion

5.1 Human coronaviruses

5.1.1 Findings in EPI I material (I)

In Study I, EPI I material collected from September 2006 to August 2008 was used. Eight-hundred seventy-eight children with acute gastroenteritis and 112 control children – including 43 children with indeterminate fever and vomiting, 33 children with ARTI, and 36 healthy children – were studied. The characteristics of the studied children are shown in Table 1 in the Materials and Methods section.

Twenty-two of 878 stool specimens (2.5%) of the children with AGE were positive for HCoVs. In addition, two control children – one in the indeterminate fever and vomiting group and one in the group of healthy children – had HCoV RNA in their stools. The difference in the proportion of HCoV-positive stool samples between the AGE and separate control groups was not statistically significant (Fisher's exact test, p=0.935). Even though the control cases were handled as a single group, the difference between the AGE cases and controls remained statistically insignificant (Fisher's exact test, p=1.000) (Table 4).

Table 4. The number of HCoV positive stool samples in the AGE group (children with acute gastroenteritis) and controls (children with indeterminate fever and vomiting, children with ARTI and healthy children combined).

	AGE group	Controls
HCoV-positive	22 (2.5%)	2 (1.8%)
HCoV-negative	856 (97.5%)	110 (98.2%)

All commonly circulating HCoV types were found: HCoV-OC43 in 12 cases, HCoV-HKU1 in six, HCoV-229E in two, and HCoV-NL63 in four cases. The seasonality of the findings is shown in Figure 4.

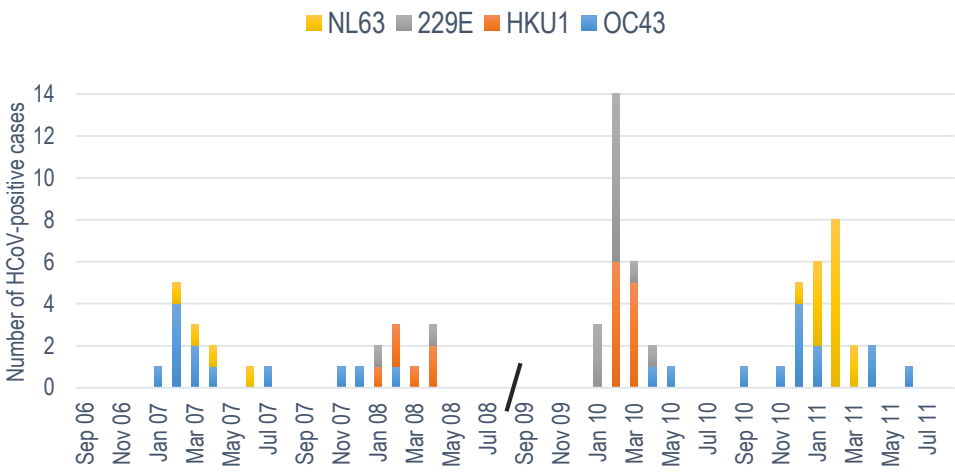


Figure 4. Seasonality of HCoV findings. EPI I and EPI II materials combined. Notice that no samples were collected between September 2008 and August 2009 (black slash).

When comparing children with HCoV-positive stool samples to those with HCoV-negative stool samples, there were no statistically significant differences in proportion of males and females (χ^2 test, p=0.837) or distribution in age groups (age groups: <6 months, 6-24 months, 2-5 years, >5 years) (Fisher's exact test, p=0.201). In addition, the proportion of stool samples positive for other pathogens (rotavirus, norovirus, sapovirus, or adenovirus) was similar in cases with a HCoV-positive stool sample compared to cases with a HCoV-negative stool sample (χ^2 test, p=0.662).

In four of the 22 HCoV-positive stool samples (18.2%), HCoV was the single virus detected in the sample, whereas in the remaining samples rotavirus or norovirus was also detected. In addition, half of the HCoV-positive children had symptoms of respiratory tract infection at the same time as AGE, or respiratory symptoms had been present before the symptoms of AGE. Children with HCoV as a single pathogen did not differ from the HCoV-positive children with mixed infections by gender (Fisher's exact test, p=1.000) or the proportion of positive findings in the different age groups (Fisher's exact test, p=0.267). None of the detected HCoVs

were significantly more common in the single cases compared to the cases with co-detection of rotavirus or norovirus (Table 5). However, it should be taken into account that the number of HCoV-positive cases was small which may influence to the statistical analysis. Representation of the HCoV-positive cases is presented in Table 6.

Table 5. Number of different HCoVs in stool samples with and without other viruses (rotavirus or norovirus).

	Single infections	Co-detection of other viruses	p-value[a]
OC43	1 (25.%)	9 (50%)	0.594
HKU1	1 (25.%)	5 (27.8%)	1.000
229E	0	2 (11.1%)	1.000
NL63	2 (50%)	2 (11.1%)	0.135
All	4	18	

[a] Calculated using Fisher's exact test

Table 6. Representation of children with HCoV-positive stool sample in the AGE group of EPI I material. Cases with HCoV as the single virus in the stools are highlighted. Respiratory symptoms included cough, rhinitis, tonsillitis, otitis media, pneumonia, and laryngitis. (yrs=years, mo=months)

Sample date	Age	Sex	HCoV type	Other viruses in stool	Respiratory symptoms
24/1/2007	**17 mo**	**F**	**OC43**	**none**	**yes**
5/2/2007	2 yrs 3 mo	M	OC43	norovirus	none
15/2/2007	12 mo	F	OC43	norovirus	yes
19/2/2007	3 yrs 5 mo	F	OC43	norovirus	none
3/3/2007	13 mo	F	OC43	norovirus	yes
26/3/2007	2 yrs 3 mo	F	NL63	rotavirus	none
1/4/2007	**17 mo**	**M**	**NL63**	**none**	**none**
1/2/2007	2 yrs 1 mo	M	NL63	norovirus	none
7/4/2007	13 mo	M	OC43	norovirus	yes
1/3/2007	9 mo	M	OC43	norovirus	none
26/6/2007	**2 yrs 7 mo**	**F**	**NL63**	**none**	**yes**
24/11/2007	3 yrs 9 mo	M	OC43	rotavirus	yes
25/12/2007	2 yrs 3 mo	F	OC43	rotavirus	yes
7/1/2008	18 mo	M	229E	rotavirus	none
21/1/2008	17 mo	M	HKU1	norovirus	yes
11/2/2008	2 yrs 5 mo	M	OC43	rotavirus	none
21/2/2008	17 mo	F	HKU1	norovirus	none
23/2/2008	10 mo	M	HKU1	rotavirus	none
24/3/2008	**6 yrs 3 mo**	**M**	**HKU1**	**none**	**yes**
9/4/2008	21 mo	M	HKU1	rotavirus	yes
21/4/2008	2 yrs 6 mo	M	229E	rotavirus	yes
28/4/2008	16 mo	F	HKU1	rotavirus	none

The findings of Study I show that all commonly circulating HCoVs can be found in the stool samples of children needing hospital care for AGE. However, some children in the control groups also harbored HCoVs in their stools, and the difference in the detection rates between the AGE cases and the controls was not statistically significant. In addition, in most HCoV-positive AGE cases there was either rotavirus or norovirus in the same stool sample, probably explaining the

symptoms of AGE. Because half of the HCoV-positive children also had symptoms of respiratory infection, the origin of the HCoV in the stool samples could be the respiratory tract, where HCoVs are known to multiply and cause infection. To further investigate this possibility, both stool and nasal swab samples were studied in Study II.

5.1.2 Simultaneous detection of HCoVs in stool samples and nasal swab samples of EPI II study (II)

EPI II material was used in Study II. This material comprised 955 cases of hospitalized children divided into three groups: the AGE group, the ARTI group, and the AGE/ARTI group. Details of the material are described in the Materials and Methods section.

Both stool and nasal swab samples of all 955 children were studied, and commonly circulating HCoVs were detected in 19 stool samples (2.0%) and 50 nasal swab samples (5.2%). In all but two cases with HCoV-positive stool samples, the corresponding nasal swab sample was positive for the same HCoV. None of the 20 available serum samples of HCoV-positive children (positive stool and/or nasal swab) was positive for HCoV RNA. When the HCoV-positive children (i.e. the stool and/or nasal swab sample was positive) were compared to HCoV-negative children, there were no statistically significant differences in proportion of males and females (χ^2 test, $p=0.056$) or proportions of cases in the different age groups (χ^2 test, $p=0.631$).

The seasonality of the findings is shown in Figure 4. HCoV-HKU1 and HCoV-229E were detected mainly during the first season, from September 2009 to August 2010, and HCoV-NL63 and HCoV-OC43 during the second season, from September 2010 to August 2011. When comparing seasonal patterns of HCoV detections in Study II to the results of Study I, a biennial circulation of HCoVs seems probable, so in principal only one alphacoronavirus type and one betacoronavirus type circulates per season.

HCoVs were found in eight of the 172 stool specimens (4.7%) of children in the AGE group, and in six of the eight cases both stool and nasal swab specimens were positive for the same HCoV. A known gastroenteritis virus – rotavirus (three cases), norovirus (two cases), or sapovirus (one case) – was present in the stools of six of

the eight children. There was only one child with an HCoV-positive stool sample without the concomitant detection of HCoV in the nasal swab or co-detection of another virus in the stool sample. The details of the AGE cases with HCoV-positive stool samples are presented in Table 7. There were also three cases in the AGE group in which the nasal swab sample was positive but the stool sample was negative for HCoV RNA. In two of these children, norovirus was detected in the stools.

Table 7. Description of the AGE cases with HCoV-positive stool samples. Cases with HCoV as a single virus in stools highlighted. (yrs=years, mo=months)

Sample date	Age	Sex	HCoV type in stool	HCoV type in nasal swab	Other viruses in stool
7/1/2010	**2 yrs 4 mo**	**M**	**229E**	**neg**	**none**
10/1/2010	2 yrs 8 mo	M	229E	229E	rotavirus
11/3/2010	19 mo	M	229E	229E	rotavirus
11/3/2010	15 mo	M	HKU1	HKU1	norovirus
9/12/2010	2 yrs 6 mo	M	NL63	neg	sapovirus
24/1/2011	22 mo	M	NL63	NL63	rotavirus
26/1/2011	13 mo	M	NL63	NL63	norovirus
17/2/2011	**2 mo**	**M**	**NL63**	**NL63**	**none**

In the ARTI group, 24 of the 545 children (4.4%) were HCoV-positive; in 19 cases, only the nasal swab sample was positive and in five cases both the stool and nasal swab samples were positive.

Seventeen of the 238 children (7.1%) in the AGE/ARTI group were positive for HCoVs, with both stool and nasal swab samples being positive in six children and the nasal swab sample alone being positive in 11 children. In five of the six children with an HCoV-positive stool sample, another virus (calicivirus in three samples, astrovirus in one, and both in one) was co-detected in the same sample.

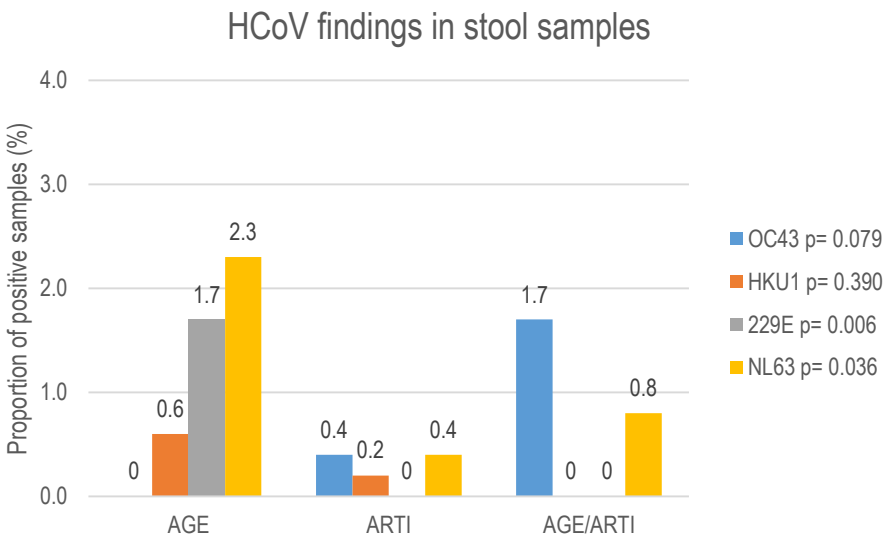
All four commonly circulating HCoVs were found and all were more commonly detected in the nasal swab samples compared to the stool samples (Table 8). The most common finding was HCoV-NL63 with 15 positive cases, followed by HCoV-OC43 (13 positive cases), HCoV-229E (13 positive cases), and HCoV-HKU1 (11 positive cases).

Table 8. Detection of HCoVs in the different sample types.

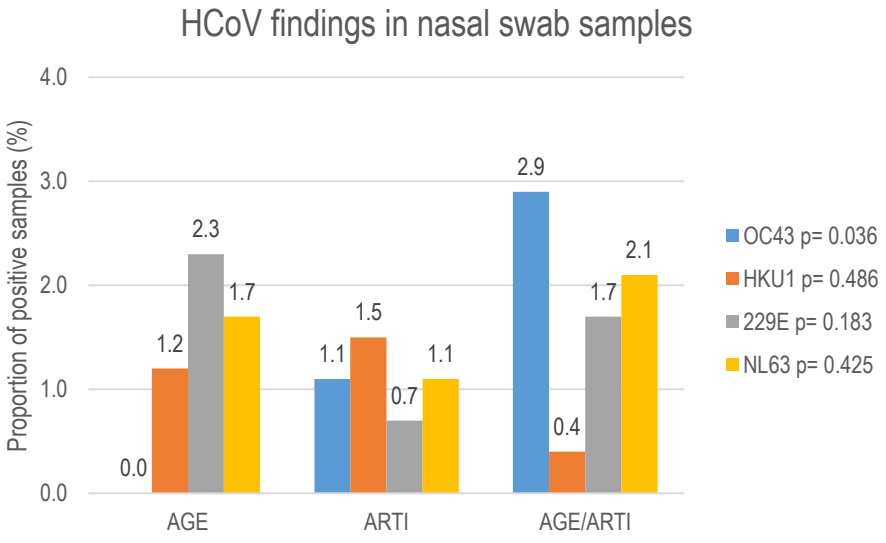
	Positive sample types			All
	Stool sample only	Nasal swab sample only	Both sample types positive	
OC43	0	7	6	13
HKU1	0	9	2	11
229E	1	10	2	13
NL63	1	7	7	15

The detection rates of different HCoVs in stool samples, nasal swab samples, and the combined results (positive stool and/or nasal swab sample) are shown in Figures 5a-c. Where only stool samples are observed, it seems that HCoV-229E and HCoV-NL63 are associated with the AGE cases, because the detection rates are higher in the AGE group compared to other study groups and a comparison of the rates gives $p<0.05$. However, most of the children with HCoV-positive stool samples also had the same HCoV in their nasal swab samples. When comparing the detection rates of different HCoVs in the nasal swab samples or combining the stool and nasal swab sample results, there is no statistically significant difference between the three study groups – excluding HCoV-OC43, which was not detected in any of the samples in the AGE group (Figures 5a-c). In addition, in most HCoV-positive stool samples there were well-known gastroenteritis viruses like rotavirus or norovirus detected in the same sample, so the AGE symptoms may have been caused by these viruses instead of HCoVs.

a)


HCoV findings in stool samples
Proportion of positive samples (%)
4.0
3.0
2.0
1.0
0.0
0
0.6
1.7
2.3
0.4
0.2
0
0.4
1.7
0
0
0.8
AGE
ARTI
AGE/ARTI
OC43 p= 0.079
HKU1 p= 0.390
229E p= 0.006
NL63 p= 0.036

b)


HCoV findings in nasal swab samples
Proportion of positive samples (%)
4.0
3.0
2.0
1.0
0.0
0.0
1.2
2.3
1.7
1.1
1.5
0.7
1.1
2.9
0.4
1.7
2.1
AGE
ARTI
AGE/ARTI
OC43 p= 0.036
HKU1 p= 0.486
229E p= 0.183
NL63 p= 0.425

c)

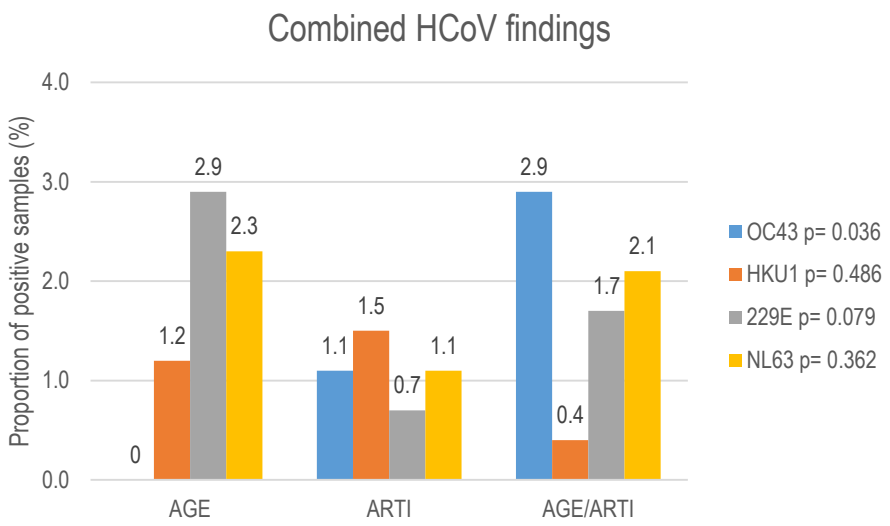


Figure 5. HCoV findings in the different study groups. a) Proportion of positive stool samples, b) proportion of positive nasal swab samples, c) combined results (positive stool and/or nasal swab sample). The proportion of the positive samples in three study groups were compared for each virus and the p-values for this were calculated using Fisher's exact test.

If we divide HCoV-positive children into those who have HCoV concomitantly in the stools and nasal swabs and those who have HCoV only in the nasal swabs, there were no statistically significant differences in the distribution of the cases in the different age groups (Fisher's exact test, p=0.457) or by gender (Fisher's exact test, p=0.173). If both the nasal swab and stool sample were positive, the nasal swab was positive in the first PCR step in most (88.7%) cases, whereas in the cases where only the nasal swab was positive, less than a half (45.5%) of the nasal swab samples were positive in the first PCR step. This difference was statistically significant (χ^2 test, p=0.005) and suggests that a higher virus load in the nasal swab is associated with the presence of the virus in the stool sample.

Study II confirmed that all commonly circulating HCoVs can be detected in the stool samples of hospitalized children. However, all HCoVs were more commonly

detected in the nasal swab samples than stool samples, and in almost all cases with an HCoV-positive stool sample, the corresponding nasal swab sample was positive for the respective HCoV. In addition, in most of the AGE cases with an HCoV-positive stool sample, there were well-established gastroenteritis viruses like rotavirus or norovirus detected in the same stool sample. This suggests that findings in stools are associated with the presence of HCoV in the respiratory tract rather than the symptoms of AGE, and the symptoms of AGE could be explained by other viruses. The connection between HCoV findings in the respiratory tract and stool samples is further strengthened because it seemed that a relatively higher virus load in the nasal swab samples (estimated by first PCR positivity) was associated with the presence of HCoVs in the stools. It cannot be concluded whether the presence of an HCoV in stools is due to swallowing the virus from the respiratory tract. However, when it is taken into account that reproduced virus particles of commonly circulating HCoVs are mainly released from the apical surface of the epithelial cells (33), and that all studied serum samples were negative for HCoVs, the possibility that the virus was spread from the respiratory tract to the gastrointestinal tract via the bloodstream seems unlikely. The infrequency of viremia in HCoV cases has also been reported by others (247). However, it has been hypothesized in one SARS-CoV study that SARS-CoVs are carried from the respiratory tract to the gut via the blood by infected circulating lymphocytes (90).

The proportion of positive cases was not significantly higher in the AGE groups compared to controls in either of the studies, so a specific association with AGE could not be made. Nevertheless, in Study II, HCoV-229E and -NL63 were more commonly found in the stool samples of AGE patients compared to ARTI patients and patients with the symptoms of both AGE and ARTI. However, the number of HCoV-229E- and -NL63-positive stool samples in AGE cases was small, three and four, respectively, and in only two cases was the respective HCoV detected in the stools alone (Table 7). In addition, the same HCoV was usually present in the respiratory tract at the same time. Even though the HCoVs in these two cases (without other viruses in the stools) may have had a role in causing the symptoms of AGE, their significance in the whole material is minimal.

The intriguing detail in the EPI I material of Study I is children whose AGE was acquired during the Pirkanmaa waterborne AGE outbreak of November and December 2007. This outbreak was caused by accidental contamination of drinking water by sewage, and it is more precisely described by Räsänen et al. (248). Fifty stool samples of these cases were available in EPI I material, and most of the samples had multiple viral and bacterial pathogens combined with a severe clinical picture (248).

However, none of these stool samples was positive for HCoVs, suggesting that the presence of HCoVs in sewage is not common or that because the main target of the virus is the respiratory tract: even massive exposure through the gastrointestinal tract does not cause gastrointestinal symptoms.

Other recently published studies concerning commonly circulating HCoVs and AGE are in agreement with our findings. In a Slovenian study where both stool and nasopharyngeal swabs were collected from children with AGE and healthy controls, there were significantly more HCoV-positive cases in the AGE group compared to the controls, but in most cases the HCoV was found in the nasopharyngeal swab samples, not in the stools. In addition, over half of the children also had symptoms of respiratory tract infection, and in the majority of the AGE cases, known gastroenteritis viruses were detected in the same stool sample. The authors concluded that HCoVs probably played only a minor role in the AGE cases of the studied children (247). In an American study, the frequency of HCoVs were studied in 479 children and adults with diarrhea, but only four HKU1-positive samples were found: most of these patients also had symptoms of respiratory infection. Again, the authors suggested only a minor role for HCoVs in AGE cases (249).

The most tempting explanation for HCoV findings in the stools of hospitalized children is that HCoVs infect the upper respiratory tract and are swallowed, because HCoVs are seldom detected only in stools without positivity in the respiratory tract. This explanation is supported by the suggestive evidence of Study II that higher amount of HCoVs in the respiratory tract is connected to the presence of HCoVs in stools. Whether commonly circulating HCoVs actually infect and replicate in the intestinal cells could be possible in theory, because, for example, the receptors for HCoV-229E and -NL63 are present in the intestinal epithelial cells (34,36,37), but so far there are no studies confirming the replication of these viruses in the intestinal cells. In SARS patients, active viral replication was seen in both the small and large intestine by EM, but the damage to the intestinal cells seemed minimal and there was no viable virus in the stool samples (89). Of the animal coronaviruses, the comprehensively studied bovine coronavirus (BCV) is capable of growing in vitro in gut organ cultures, and it has also been shown that in vivo BCV replicates on the surface of gut epithelial cells, especially in those of the lower small intestine, and it is able to cause serious changes, even a complete desquamation in the surfaces of the intestines (250). A challenge for coronaviruses in infecting the intestines is to successfully pass the acidic ventricle, because as enveloped viruses they are relatively sensitive to gastric acid. This is possible at least for SARS-CoV and BCV, but there might be variation in the resistance qualities between different coronavirus species

and strains. The stability of SARS-CoV in the environment and human specimens has been shown to be relatively strong (113) and SARS-CoV has proved to be more stable in the environment compared to HCoV-229E (114), so this may also reflect its greater ability to pass through the stomach compared to commonly circulating HCoVs. It is also possible that HCoV findings in stools are remnants of the previous infection, because it is known that HCoVs can be shed after acute infection; for example, SARS-CoV has been detected in stool samples occasionally over six weeks after the onset of the symptoms (58), and HCoV-NL63 has been detected in nasopharyngeal swabs for up to three weeks after the acute infection (251). It may also be possible that HCoV caused a mild gastrointestinal infection that predisposed the host to an infection of a more pathogenic virus like rotavirus, which further caused an infection severe enough to lead to a visit to the hospital. Even though HCoVs mostly cause mild infections that do not lead to hospitalization and thus the HCoV findings in stools could be more prevalent in children treated at home, the above-mentioned speculation is hypothetical and requires further study.

In both study materials, a roughly biennial periodicity of the HCoV infections was seen, which confirms the observations made by other study groups in Europe (101,121). This would mean that if all four commonly circulating HCoVs are to be studied, at least two epidemic seasons should be evaluated.

5.2 Human bocaviruses

5.2.1 Findings in stool samples of EPI I study (III)

In Study III, HBoV1-4 DNA was searched for from 878 stool specimens of children hospitalized for AGE and 112 stool specimens of control children, including 43 children with indeterminate fever and vomiting, 33 children with ARTI, and 36 healthy children. Details of the study material, the EPI I study, are described in the Materials and methods section.

HBoV DNA was found in 85 (9.7%) stool samples of children with AGE and in 6 (5.4%) stool samples of the control cases. The proportion of positive samples did not differ statistically significantly between the cases and the combined control groups (χ^2 test, $p=0.165$).

HBoV1 was the most common HBoV detected, accounting for 49 (5.6%) of the AGE cases. There were also two positive samples in the control groups, one (2.3%)

in a child with indeterminate fever and vomiting, and one (3.0%) in a child with respiratory tract infection. Detection rates did not differ significantly between the AGE group and control groups either when control groups were handled separately (Fisher's exact test, p=0.529) or when they were handled as one group (χ^2 test, p=0.110) (Table 9). In nine (18.4%) of the 49 HBoV1-positive cases in the AGE group, HBoV1 was detected in the stools alone, and in other cases, rotavirus, norovirus, sapovirus, or enteric adenovirus was detected in the same sample. Other viruses were detected at approximately similar rates in the HBoV1-positive and HBoV1-negative stool samples in the AGE group (81.6% versus 77.8% respectively; χ^2 test, p=0.599). HBoV1 was most often detected in children aged 6-24 months, but the differences between age groups (age groups: <6 months, 6-24 months, 2-5 years, >5 years) were not statistically significant (χ^2 test, p=0.203) (Figure 6). For some reason, girls had more HBoV1-positive samples compared to boys (7.0% versus 3.8%, respectively; χ^2 test, p=0.029).

HBoV2 was detected in 29 stool specimens (3.3%) from the children with AGE and in two samples from the control children, including one sample (2.3%) from the indeterminate fever and vomiting group, and one sample (2.8%) from the group of healthy children. Detection rates did not differ significantly between the AGE and control groups, either when control groups were handled separately (Fisher's exact test, p=0.949) or when they were handled as a single group (Fisher's exact test, p=0.567) (Table 9). Twenty-one (72.4%) of the 29 HBoV2-positive cases were co-detections with other viruses (including rotavirus, norovirus, sapovirus, and enteric adenovirus) while in eight AGE cases (27.6%) HBoV2 was detected alone. One stool sample harbored both HBoV1 and HBoV2: the same sample was also positive for rotavirus. There was no significant difference in the detection rates of well-established gastroenteritis viruses between HBoV2-positive and HBoV2-negative stool samples in the AGE group (rates 72.4% and 78.2%, respectively; χ^2 test, p=0.493). HBoV2 was detected most often in children aged 2-5 years (Fisher's exact test, p=0.041) (Figure 6). Three percent of the girls and 3.2% of the boys were positive for HBoV2; the difference was not statistically significant (χ^2 test, p=1.000).

Eight stool samples (0.9%) from children with AGE were positive for HBoV3 compared to one positive sample (2.3%) from the indeterminate fever and vomiting group, and one sample (3.0%) from the group of children with respiratory tract infection. The differences in the detection rates between AGE cases and separate or combined control groups were not statistically significant (Fisher's exact test, p=0.260 and p=0.315, respectively) (Table 9). Seven (87.5%) of the eight

HBoV3-positive samples from the AGE group harbored known gastroenteritis viruses (precisely rotavirus, norovirus, or both) in the same sample; in HBoV3-negative samples, well-known gastroenteritis viruses were detected in 77.9% of the cases. The distribution of HBoV3-positive samples in the different age groups is shown in Figure 6. HBoV3 was detected in 0.7% of the girls and in 1.3% of the boys (Fisher's exact test, p=0.527).

HBoV4 was not detected in any of the samples.

Table 9. HBoV-positive cases in the different study groups. (The proportion of positive samples in different study groups were compared and p-values for this comparison calculated using Fisher's exact test.)

Virus	Number of positive samples in the study groups				
	AGE* (N=878)	Fever** (N=43)	Resp.*** (N=33)	Healthy (N=36)	p-value
HBoV1	49 (5.6%)	1 (2.3%)	1 (3.0%)	0	*0.529*
HBoV2	29 (3.3%)	1 (2.3%)	0	1 (2.8%)	*0.949*
HBoV3	8 (0.9%)	1 (2.3%)	1 (3.0%)	0	*0.260*

* children with acute gastroenteritis

** children with unknown fever and vomiting

*** children with respiratory tract infection

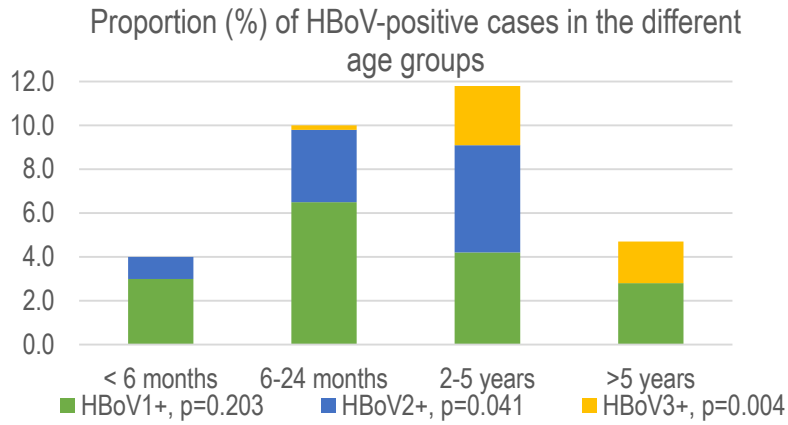


Figure 6. HBoV-positive findings in the different age groups of the EPI I study. (The p-values were calculated by using χ^2 test for HBoV1 and Fisher's exact test for HBoV2 and HBoV3.)

The detection rates for HBoV1 and HBoV2 did not differ significantly between the seasons (data not shown), but HBoV3 was detected only during the first season (September 2006 – August 2007). Seasonal distribution of HBoV-positive cases is shown in Figure 7.

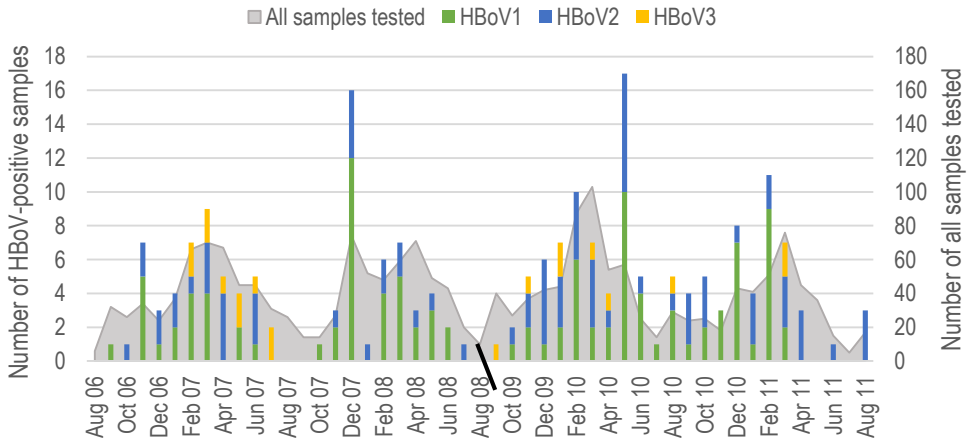


Figure 7. Seasonality of HBoV findings and all samples studied. EPI I and EPI II materials combined. Notice that no samples were collected between September 2008 and August 2009 (black slash).

Overall, Study III showed that HBoVs – with the exception of HBoV4 – are relatively common findings in the stool specimens of children requiring hospital care in Finland. However, in addition to the stool samples of children with AGE, HBoVs were also detected in the stools of children without a diagnosis of gastroenteritis, and in most of the HBoV-positive AGE cases (81.6% of HBoV1 cases, 72.4% of HBoV2 cases, and 87.5% of HBoV3 cases) there were other well-established gastroenteritis viruses like rotavirus or norovirus in the same stool sample, making it difficult to estimate the impact of HBoVs on the symptoms. It should be mentioned that after Study III was published, more intensive testing of adenoviruses were done for the material and two of the adenoviruses co-detected with HBoV1 were found to be non-enteric adenoviruses, and thus the co-detection rate of HBoV1 is slightly lower (81.6% versus 85.7%) than reported in the article. However, even after this change in the co-detection rate, HBoV2 was the HBoV with lowest co-detection rate.

Study III produced information about the epidemiology of HBoVs in Finnish children, and even though none of the HBoVs could be associated with AGE, there were however some features that seem to require further evaluation. What was the meaning of the single HBoV infections? Could the putative, long HBoV shedding in stools influence the results? Were the control groups big enough or otherwise optimal? As especially HBoV1 is also commonly found in respiratory samples, simultaneous screening of both stool and respiratory samples seemed to be necessary to further assess the impact of HBoVs, and serum specimens would be needed for specifying acute infections.

5.2.2 HBoVs in stool and nasal swab samples of EPI II study (IV)

In Study IV, the EPI II material was used, and in addition to 955 stool specimens and an equal number of nasal swab specimens, serum samples were available for approximately one fourth of the patients. The material is more precisely described in the Materials and Methods section.

HBoV1 was found in only three (1.7%) of the 172 cases in the AGE group. One child had HBoV1 in both the nasal swab and stool samples, in another child HBoV1 was detected only in stools, and in a third child HBoV1 was detected only in the nasal swab. In both HBoV1-positive stool samples, another virus – precisely

norovirus or sapovirus – was also detected. In the ARTI group, HBoV1 was found in 34 (6.2%) of the 545 patients. In 18 cases (52.9% of the 34 positive cases), both sample types were positive, in 11 cases HBoV1 was detected only in the stools, and in five cases it was only in the nasal swab. In the AGE/ARTI group, HBoV1 was detected in 22 cases (9.2%): in 12 children both the stool and nasal swab were positive, in six children only the stool sample was positive, and in four children only the nasal swab sample harbored HBoV1. The difference in the detection rates between study groups was statistically significant (χ^2 test, $p=0.008$). HBoV1 was thus most often detected in children with respiratory symptoms, the highest detection rate being in children with both respiratory and gastrointestinal symptoms (AGE/ARTI group). Because the AGE/ARTI group comprised children with diverse combinations of gastrointestinal and respiratory symptoms, the separate cases need to be analyzed more closely. Of the 22 HBoV1-positive cases in the AGE/ARTI group, the principal diagnosis was gastroenteritis (ICD10 codes A01-A09) in nine cases, and in six of these, another virus (rotavirus, adenovirus, or astrovirus) was co-detected. In addition, in all three cases without well-known gastroenteritis viruses in stools, HBoV1 was detected only in the nasal swab sample. Thus, if prominent gastrointestinal symptoms were present, the cause was probably other than HBoV1. Overall, HBoV1 was more commonly detected in the stools compared to the nasal swab samples (49 and 41 positive cases respectively). The proportions of positive samples in the different study groups is shown in Figure 8.

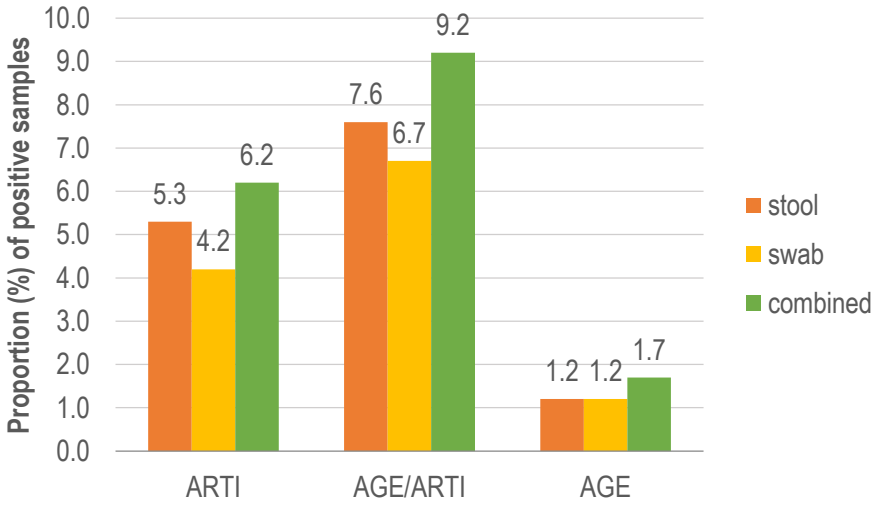


Figure 8. Proportion of HBoV1-positive samples in the different study groups. (combined = stool and/or nasal swab sample positive)

HBoV2 DNA was detected in ten patients (5.8%) in the AGE group. In all of these cases, HBoV2 was found in the stools and in the nasal swab sample of one patient as well. Nine of the ten stool samples contained other viruses (rotavirus in five cases, norovirus in three cases, and enteric adenovirus in one case), and HBoV2 was detected alone in only one sample. In the ARTI group, HBoV2 was found in 28 cases (5.1%), in all these cases, HBoV2 was detected in the stools, and in two patients also in the nasal swab. In the AGE/ARTI group, 13 stool specimens (5.5%) harbored HBoV2 DNA, whereas all nasal swab samples were negative. In eight of the 13 cases, AGE was the main diagnosis, and other viruses (norovirus in three cases, enteric adenovirus in one case, and both rotavirus and sapovirus in one case) were co-detected with HBoV2 in five of these children. The difference in the detection rates between study groups was not statistically significant (χ^2 test, p=0.941). An interesting observation concerning HBoV2-positive cases was that some of the children harbored HBoV2 in two consecutive samples, with the time period between samples varying from three weeks to four months. More than one sample was studied because these children were recruited to the study more than once due to the long study period. In all these "shedding" cases, the principal diagnosis was respiratory infection. The proportions of positive samples in the different study groups is shown in Figure 9.

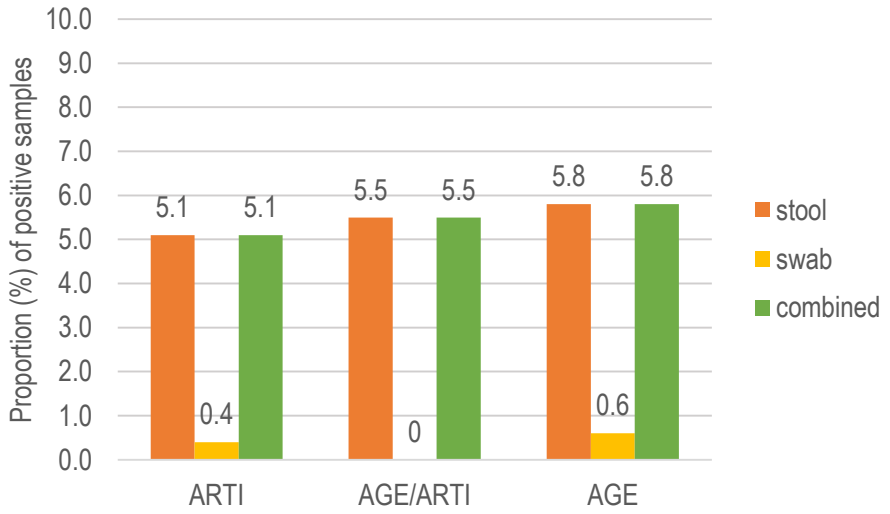


Figure 9. Proportion of HBoV2-positive samples in the different study groups. (combined = stool and/or nasal swab sample positive)

HBoV3 was found in nine stool specimens, and none of the nasal swabs was positive. The proportions of positive cases did not differ statistically significantly between study groups (Fisher's exact test, p=1.000). Rotavirus or norovirus was detected in all HBoV3-positive stool samples from the children in the AGE or AGE/ARTI groups. The proportion of positive samples in the different study groups is shown in Figure 10.

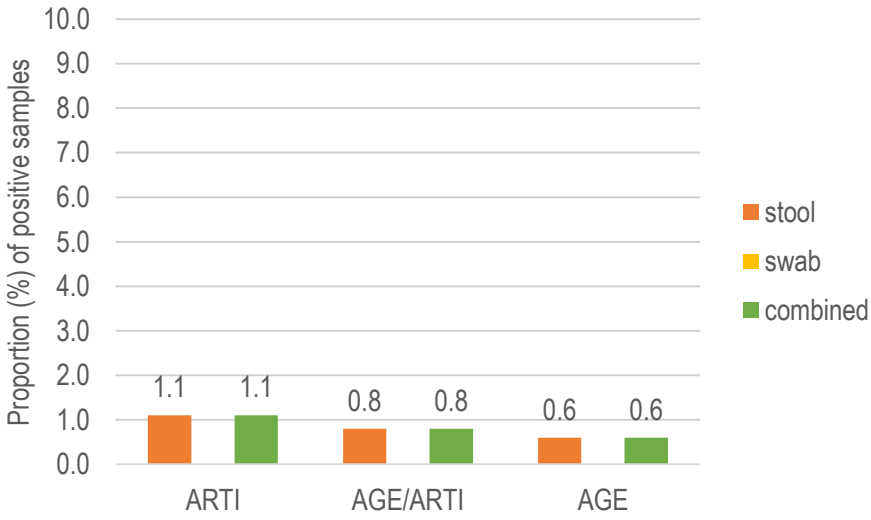


Figure 10. Proportion of HBoV3-positive samples in the different study groups. (combined = stool and/or nasal swab sample positive)

HBoV4 was not detected in any of the samples.

HBoV1 was detected in 5.0% of females compared to 6.9% of males (χ^2 test p=0.269), HBoV2 was detected in 6.1% of females compared to 4.9% of males (χ^2 test p=0.458), and HBoV3 was detected in 1.4% of females compared to 0.7% of males (Fisher's exact test, p=0.319). The distribution of positive cases in the different age groups is shown in Figure 11, and as shown, HBoV1 and HBoV2 were more common in children aged 6-24 months compared to other age groups. The seasonality of the HBoV findings is illustrated in Figure 7.

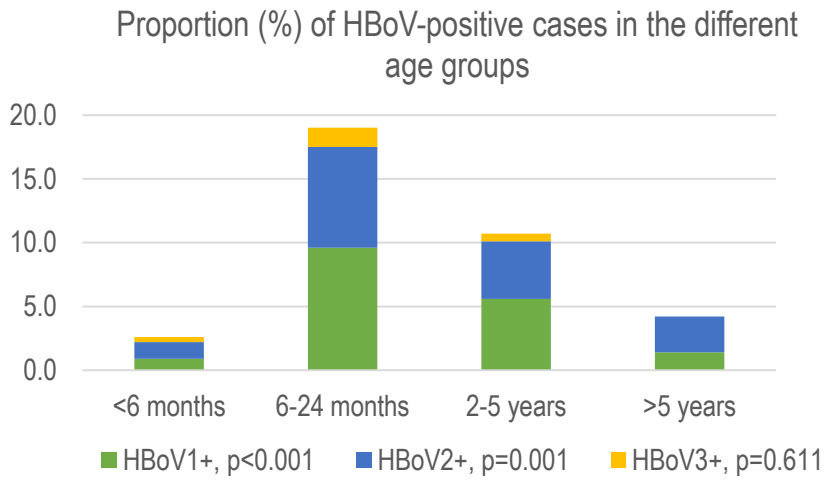


Figure 11. HBoV-positive findings in the different age groups of the EPI II study. (Proportions of HBoV-positive cases in different study groups were compared. p-values for this comparison were calculated by using Fisher's exact test.)

Based on the PCR findings, none of the HBoVs was convincingly associated with AGE when the different study groups were compared. HBoV1 instead was associated with respiratory symptoms, which is in line with being a cause of respiratory tract infections.

Because PCR-based methods are not able to distinguish between past and acute infection reliably, and because symptomless shedding is known to occur at least in some portion of HBoV infections, serum samples were collected for antibody testing. Unfortunately serum samples were obtained for only 288 of the study children. Of the 117 HBoV-positive children, an acute-phase serum sample was available for 30 children and convalescent-phase sera for five of those. IgG and IgM antibodies were studied from the serum samples; in addition, quantitative PCR was done to detect viremic cases. HBoV IgM antibodies were detected in ten acute-phase sera; in two cases IgG seroconversion was also observed in paired sera. Eight of the ten sera were also viremic, with full concordance with an HBoV type in the serodiagnosis. In addition, two serum samples harbored HBoV1 DNA and one sample harbored HBoV3 DNA without serological signs of acute infection. Separate cases are shown in Table 10. Acute HBoV2 infection was detected in two children with AGE and one child in the AGE/ARTI group, but in all these cases norovirus was also detected in stools and may have caused the symptoms.

Table 10. HBoV infections with IgM antibodies or DNA positivity in serum.

					PCR findings			
Study group	Gender	Age	Dg*	IgM	stool	swab	serum	other**
AGE	F	16 mo	A08.4	**HBoV2**	**HBoV2**	neg	**HBoV2**	**norovirus**
AGE	M	11 mo	A09	**HBoV2**	**HBoV2**	neg	**HBoV2**	**norovirus**
ARTI	M	23 mo	J21.9	**HBoV1**	**HBoV2**	neg	neg	neg
AGE/ARTI	M	19 mo	A09	**HBoV1**	**HBoV1**	neg	**HBoV1**	**astrovirus**
AGE/ARTI	M	20 mo	J22	**HBoV2**	**HBoV2**	neg	**HBoV2**	**norovirus**
AGE/ARTI	M	18 mo	A08.4	**HBoV1**	neg	**HBoV1**	**HBoV1**	**norovirus**
AGE/ARTI	M	23 mo	J21.9	**HBoV1**	**HBoV1**	**HBoV1**	**HBoV1**	neg
AGE/ARTI	M	2 yrs 9 mo	A08.4	**HBoV1**	**HBoV1**	**HBoV1**	**HBoV1**	**rotavirus**
AGE/ARTI	F	3 yrs 4 mo	J18.9	**HBoV1**	**HBoV1**	**HBoV1**	**HBoV1**	neg
AGE/ARTI	F	4 yrs	J18.9	**HBoV1**	**HBoV1**	neg	neg	neg
AGE/ARTI	M	5 mo	J21.9	neg	**HBoV3**	neg	**HBoV3**	**norovirus**
AGE/ARTI	M	8 mo	J04.0	neg	**HBoV1**	neg	**HBoV1**	**norovirus**
AGE/ARTI	M	12 mo	J18.9	neg	**HBoV1**	**HBoV1**	**HBoV1**	**norovirus**

*diagnosis by ICD-10 codes (in the AGE/ARTI group this is the principal diagnosis/diagnosis of dominating symptoms): A08.4, unspecified viral intestinal infection; A09, other gastroenteritis and colitis of infectious and unspecified origin; J22, unspecified acute lower respiratory infection; J21.9, unspecified acute bronchiolitis; J18.9, unspecified pneumonia; J04.0, acute laryngitis

** other viruses, including rotavirus, norovirus, sapovirus, astrovirus, and enteric adenovirus, detected simultaneously in stools

Study IV confirmed the widespread circulation of HBoVs in Finnish children. In line with Study III and the observations of serological studies (167,182), HBoV1 was found to be the most prevalent HBoV, followed by HBoV2 and HBoV3. HBoV4 is in general a rare virus, and so far it has not been detected in Finland ((177), in addition to studies of this dissertation).

The purpose of Study IV was to evaluate the disease associations of different HBoVs, especially the connection to acute gastroenteritis. This association was not found and co-detections with well-known gastroenteritis viruses in the majority of HBoV-positive cases diminished the significance of the HBoV findings in AGE cases. Even in the acute HBoV infections, which were determined by serology or detection of HBoV DNA in the serum, the causative pathogen was probably one other than HBoV. However, based on the findings in the different sample types, HBoV2 and HBoV3 seem to be enteric viruses, whereas HBoV1 was more widely found in both sample types and thus may also cause a wider spectrum of diseases.

In a study by Proenca-Modena et al., diarrhea was more common in HBoV1-positive ARTI patients compared to HBoV1-negative ARTI patients, but viruses were not studied from the stools (215). In our study, HBoV1 was most common in the AGE/ARTI group, but more detailed analysis showed that significant gastrointestinal symptoms were again probably caused by other viruses, or the HBoV1 was detected in these cases only in the nasal swab sample. An interesting detail was that HBoV1, which is known mainly as a respiratory virus, was found in the stools more often than in the nasal swab samples. It therefore seems that HBoVs in the stools, including HBoV1, are not "just" swallowed from the respiratory tract. As a comparison, animal bocavirus BPV reproduce at first in the tonsils and intestinal tract and after that it spreads via the blood stream to lymphoid tissue (252). This kind of strategy could be an intriguing possibility for HBoV1 too, because HBoV1 DNA and mRNA have been detected in adenoids (163,253), and viremia is commonly observed especially in acute infections (173). The pathogenetic mechanism has been studied only for HBoV1 in HAE cell cultures, in which it caused disruption of tight junctions, epithelial cell hypertrophy, and loss of cilia (160,161). Whether this kind of destruction could also happen in gut epithelial cells is currently not known. For comparison, BPV causes damage in the crypt cells of the small intestine, which leads to villus atrophy and fusion (252). However, amino acid identity in the VP region between BPV and HBoV1 is only 42% (17), which may cause significant differences in the tissue tropism of these related viruses.

So far, HBoV2 has had the strongest link with AGE in children, and it has even been proposed to fill the diagnostic gap in the AGE cases of childhood (18). The studies involving HBoV2 have been, however, somewhat conflicting and the causal role of HBoV2 in AGE has remained without confirmation (224,227). In our study, the comparison of study groups did not show significant differences and even acute HBoV2 infections had the involvement of norovirus, a well-established gastrointestinal pathogen.

Because HBoVs are commonly detected together with other viruses in both the respiratory and gastrointestinal tracts, this raises interesting questions. Does an HBoV need a preceding infection caused by another virus to facilitate its own multiplication? For example, a helper virus is required for reproduction of adeno-associated virus, another virus from the parvovirus family that infects humans (133). Does an HBoV infection enhance the opportunity of infection by other viruses, as has been proposed to happen after HBoV1 infection in the respiratory tract (160)? Alternatively, are the recurrent co-detections with other viruses just a consequence of long-term shedding of HBoVs in excretions after acute infection?

In such a case, how long does the shedding take place, and in what situations does it occur? Definitive answers to these questions do not yet exist.

The determination of causality of a virus to a certain disease is not easy. Over recent decades, the development of metagenomics and the general increase in the analytical sensitivity of molecular methods have greatly improved the diagnostics of infectious diseases, but at the same time they have brought challenges. How does one distinguish the clinically relevant infections from asymptomatic shedding, and when several pathogens are detected in the same sample, how does one assess the role of individual microbes? These challenges were also dealt with in this dissertation. Different kinds of guidelines and modifications of Koch postulates have been proposed to ease the determination of causation (254). Molecular guidelines for establishing microbial disease causation by Fredricks and Relman are summarized in Table 11 (254). It is clear that several studies are needed to assess all aspects of these guidelines. The studies of this dissertation focused on criteria 1. HBoV2 was detected relatively commonly in AGE cases and the principal detection site, stools, was related to the GI tract, so it can be said that HBoV2, at least partly, fulfilled criteria 1. HBoV3 was detected only in stools, but detection rates were low, indicating weaker evidence for criteria 1. HBoV1 was present in both stools and nasal swabs of AGE patients, in a number comparable to HBoV2. HCoVs, on the other hand, were detected relatively seldom in AGE patients and were principally found in respiratory samples, indicating weak evidence for criteria 1.

Table 11. Molecular guidelines for establishing microbial disease causation by Fredricks and Relman (254) (condensed from the original article).

1) Nucleic acid (NA) sequence of the putative pathogen should be present in most cases of a disease and preferentially in those sites known to be diseased and not in those that lack the disease
2) Copy numbers of NA should be fewer or absent in cases without disease
3) With recovery, the copy numbers should decrease and become undetectable
4) Causal relationship is more probable if detection of NA predates the disease and copy numbers correlate with disease severity
5) The proposed causality should be in line with the known biological characteristics of that microbe group
6) The impact of NA to tissue should be studied at the cellular level
7) All results should be reproducible

Taken together, neither of the studies confirmed the association between HBoVs and AGE. HBoV1 was associated with respiratory symptoms and HBoV3 did not show a connection with any disease entities and the actual number of HBoV3-positive cases was small. HBoV2 has most often been linked with AGE, but in these studies, the association was not found. Whether HBoVs actually multiply in the gastrointestinal tract could not be determined in this study, and so far there are no suitable cell models for HBoV2 and HBoV3. In addition, because both study materials were hospital-based, the role of HBoVs in milder AGE cases not requiring hospital-care could not be assessed. To better elucidate the relation of HBoVs and the gastrointestinal tract, permissive cell lines are needed for pathogenetic studies. In addition, longitudinal studies focusing on stool samples and symptoms of gastrointestinal infection should be conducted to evaluate the temporal connection of HBoV infection to gastrointestinal symptoms.

Finally, there are some limitations in the studies. Samples of healthy children were studied only in the EPI I material, and even in this material the number was small, which may have influenced the statistical analyses. However, in Study IV we had large groups of children with ARTI and AGE to compare. There are also some disadvantages in cross-sectional studies, like both EPI studies, when studying pathogens that are common and cause reinfections, and may also be shed after acute infection. HBoVs have been shown to match the aforementioned features and thus longitudinal studies with a sampling interval short enough would be better when assessing the temporal association between HBoV findings and symptoms (255). In addition, because both EPI studies were hospital-based, the symptoms of studied children had usually lasted for some time before sampling and thus the peak viral load of the early disease may have been missed. Another limitation may have been the use of a qualitative PCR method for stool and nasal swab samples, because the viral load could not be quantified and a comparison of virus loads between study groups could not be done. In previous studies, a high HBoV1 load has correlated with the acuteness of infection and absence of other viruses, but only HBoV1 and respiratory samples were investigated (173,186,191). HBoV copy numbers between stool samples from patients with gastroenteritis and healthy controls have been compared, but without significant differences (224,226,227). In addition, calculations of viral load in sample types like stools and nasal swabs, compared to, for example, blood samples, should be interpreted with caution, because the sampling technique in these sample types is difficult to standardize. This is why serum samples are precious when determining the acuteness of HBoV infection, but unfortunately these sample types were obtained for only a small proportion of the children in our

studies. Even the serology may not give the final answer, because a recent study by Kantola et al. showed that in heterologous secondary infections by another HBoV type, the rise in antibody levels is low (167). It has also been proposed that HBoV2-4 may cause superficial infection without significant IgG or IgM response (166).

The strengths of our study included large number of samples of AGE cases in both materials and the simultaneous collection of nasal swab specimens and stools in the EPI II material. Well-established gastrointestinal viruses, especially rotaviruses and noroviruses, were comprehensively studied, which is important in assessing the significance of HBoV findings.

6 Conclusions and future prospects

The aim of this dissertation was to investigate the occurrence and significance of HCoVs and HBoVs in children requiring hospital care because of AGE. Primarily stool samples were studied, but because of the formerly established associations of HCoVs and HBoV1 with respiratory tract infections, nasal swab samples and children with ARTI were also included in the second study material.

Neither human coronaviruses nor human bocaviruses emerged as significant pathogens in the AGE of hospitalized children in this dissertation. Human coronaviruses have long been known as common respiratory viruses, and the studies of this dissertation are in line with this role, because if an HCoV was detected in stools it was almost invariably found simultaneously in the respiratory tract, suggesting that stool findings were remnants of the virus production in the respiratory tract. In addition, if an HCoV was found in the stool sample of a child with AGE, there were well-established gastroenteritis viruses, like rotavirus or norovirus, in the same sample, thus more likely explaining the symptoms. Compared to HCoVs, HBoV DNA in the stools was not similarly linked with the presence of the virus in the respiratory tract. HBoV2 and HBoV3 were mainly found in feces, and could be regarded as enteric viruses, while HBoV1 was detected in both stools and nasal swabs. However, even the enteric HBoVs were found at similar rates in children with and without AGE, and they were seldom detected in the stools of AGE patients without well-established gastroenteritis viruses, which does not support their role as a significant gastrointestinal pathogen. The detection of HBoV1 in stools was rather a sign of respiratory tract infection than AGE. However, because of the nature of HBoVs as viruses capable of causing long shedding or persistent infections, and the limited number of serum samples obtained for serology in this dissertation, a longitudinal study with a short sampling interval and better availability of serum samples may be required to further determine the role of HBoVs in AGE of children.

The gap in the AGE diagnostics could not be convincingly filled with HCoVs and HBoVs, but because metagenomic methods are more generally used, it is reasonable to assume that more viruses will also be found in stool samples. When assessing the impact and causality of these putative new viruses with clinical entities,

new strategies should be evaluated. Because PCR-based methods are seldom sufficient alone, and as serologic methods also have problems – for example, due to the OAS phenomenon or the lack of systemic antibody response – technologies detecting virus-specific transcriptional profiles might be a welcome addition to the researcher's toolbox. The simultaneous detections of the microbe itself and the response it triggers in the infection site and systemically could solve some of the challenges the sensitive nucleic-acid detection methods have brought about.

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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.

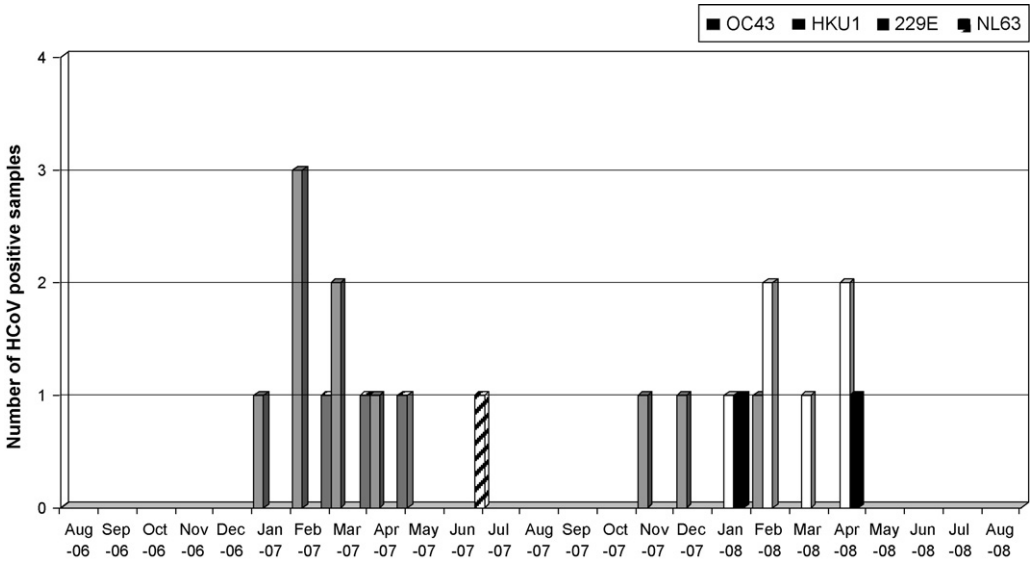

Fig. 1. Seasonal distribution of human coronavirus positive stool specimens of children with acute gastroenteritis.

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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Conflict of interest

None declared.

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Commonly circulating human coronaviruses do not have a significant role in the etiology of gastrointestinal infections in hospitalized children

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ABSTRACT

Background: Human coronaviruses (HCoVs) OC43, 229E, NL63 and HKU1 are common causes of respiratory infections. Over the years, it has been proposed that HCoVs play a possible role in gastrointestinal infections.
Objectives: To assess the role of HCoVs in acute gastroenteritis (AGE) in children.
Study design: Study was conducted at Tampere University Hospital over 2 years. Both stool and nasal swab samples were collected from 172 children with AGE, 545 with acute respiratory tract infection (ARTI) and 238 with symptoms of both. The samples were tested for HCoVs by RT-PCR.
Results: HCoVs were detected in 52 (5.4%) children: in 6.4% of those with AGE, 4.4% with ARTI and 7.1% with symptoms of both. HCoVs OC43, HKU1, 229E and NL63 were encountered in 13, 11, 13 and 15 cases, respectively. HCoVs were detected simultaneously in stool and nasal swab samples in 17 children, in nasal swabs alone in 33 children, and in the stools alone in two children. HCoVs were present in the stools of eight (4.7%) of the 172 children with AGE; in six of these cases, the nasal swab sample was also positive for the respective HCoV. Additionally, in six of the eight cases, the stool sample contained either rotavirus or calicivirus.
Conclusions: HCoVs can be detected in the stools of children with AGE, but usually together with well-known gastroenteritis viruses, and concomitantly in the respiratory tract. It appears that commonly circulating HCoVs do not have a significant role in the AGE of children admitted to hospital.

1. Background and objectives

Human coronaviruses (HCoV) cause respiratory infections of varying severity. OC43 and 229E, the "common cold viruses", have been known since the 1960s [1–4], and they have been found in 1.9% of children hospitalized for respiratory tract infection [5]. A more recently discovered coronavirus, NL63 [6], has been detected in 1.7–3.2% of respiratory tract samples of children hospitalized with acute respiratory tract infection (ARTI) [5,7–9], including acute laryngitis [5,7,9]. Another recently discovered human coronavirus, HKU1 [10], was detected in 2.95% of children hospitalized for upper or lower respiratory tract infections [11]. It seems that all of these HCoVs may cause respiratory tract infections in children that are severe enough to lead to hospitalization, but none of them seems to be more pathogenic than any other [12].

HCoVs can also cause serious disease, as shown by SARS-CoV experience [13–16] and the recently discovered MERS-CoV, which causes severe respiratory infection and renal dysfunction [17]. Both of these coronaviruses are of animal origin and are not well established in humans.

Electron microscope (EM) studies in the 1970s detected coronavirus-like particles in the stools of children with acute gastroenteritis (AGE), and the existence of enteric coronaviruses was proposed [18]. The possibility that HCoVs could have a role in gastrointestinal infections is supported by findings that some animal coronaviruses are "pneumoenteric" and capable of causing both gastrointestinal and respiratory tract infections [19]. When SARS-CoV was discovered in 2003, it was noted that 23.6–73% of SARS patients had diarrhea [16,20,21], SARS-CoV RNA could be detected in the patient's stools [14,16,21], and SARS-CoV could be isolated by culture in the intestinal tissues [21].

Gastrointestinal symptoms may also occur in patients with non-SARS-CoVs, but usually HCoVs have been studied only from respiratory samples [22] or, if found in stool samples, the

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Table 1
Characteristics of the study groups.

	N	Male (%)	Age (median)	Number of children in the different age groups				Number of cases per season	
				<6 months	6–24 months	2–5 years	>5 years	2009–2010	2010–2011
All patients studied	955	62.3	14 months	224 (23.5%)	481 (50.4%)	178 (18.6%)	72 (7.5%)	559 (58.5%)	396 (41.5%)
AGE[a] group	172	61.6	20 months	31 (18.0%)	63 (36.6%)	48 (27.9%)	30 (17.4%)	92 (53.5%)	80 (46.5%)
ARTI[b] group	545	62.9	13 months	156 (28.6%)	275 (50.5%)	93 (17.1%)	21 (3.9%)	352 (64.6%)	193 (35.4%)
AGE/ARTI[c] group	238	61.3	13 months	37 (15.5%)	143 (60.1%)	37 (15.5%)	21 (8.8%)	115 (48.3%)	123 (51.7%)

[a] Acute gastroenteritis.
[b] Acute respiratory tract infection.
[c] Symptoms of both AGE and ARTI.

simultaneous presence of gastrointestinal and respiratory symptoms has made the interpretation of the results difficult [23,24]. In our previous study, we found all four commonly circulating HCoVs in the stool samples of children with AGE [23]. However, half of these children also had respiratory symptoms, and some of the stool samples of the control children without gastrointestinal symptoms also harbored HCoVs.

In this prospective study, we simultaneously collected stool and nasal swab samples from children with AGE and an ARTI in order to clarify whether HCoV findings in stools are actually associated with AGE.

2. Study design

2.1. Patients and samples

This study was approved by the Ethics Committee of Pirkanmaa Hospital District and conducted at Tampere University Hospital's Department of Pediatrics from September 2009 to August 2011.

Children under 16 years of age with AGE who were admitted as outpatients or inpatients, or who came down with AGE during a stay in hospital, were eligible for the study. Of children with ARTI, those admitted as inpatients were eligible. Informed consent was obtained from the parents of all children enrolled. For the analysis of the study results, the patients were divided into three groups: the AGE group (children with symptoms of gastrointestinal infection only), the ARTI group (children with symptoms of respiratory tract infection only), and the AGE/ARTI group (children with different combinations of symptoms of both AGE and ARTI). Some children were admitted to the hospital more than once during the study period, and these admissions were considered to represent separate episodes if the child had been healthy for at least 2 weeks between the admissions.

Altogether, 1610 patients were eligible for the study, but in only 955 cases were both stool and nasal swab samples available and tested for HCoVs. These 955 cases included 172 children with AGE, 545 with ARTI and 238 with symptoms of both AGE and ARTI (Table 1). Moreover, 288 acute phase serum samples were available from these 955 children.

In addition to HCoVs, all stool and nasal swab samples were examined for human bocaviruses [25], all stool samples were examined for rotaviruses [26], and the stool specimens of the patients in the AGE and AGE/ARTI groups were examined for caliciviruses (including noroviruses and sapoviruses) [26,27]. HCoV-positive stool samples were additionally tested for adenoviruses and astroviruses.

2.2. Methods

Stool specimens were diluted in phosphate-buffered saline to create 10% suspensions. Nasal swab specimens were collected in UTM-RT Mini tubes (Copan Italia, Brescia, Italy), blended, centrifuged, and used for extraction.

A QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) was employed to extract viral nucleic acid from stool suspensions, nasal swabs and sera. The nucleic acid was amplified by a two-step RT-PCR method as described previously [23]. Primers covered a part of the conserved polymerase gene region and were designed to recognize all HCoVs in the first PCR step, and more specifically group 1B (229E and NL63, now classified as alphacoronaviruses), group 2A (OC43 and HKU1, now lineage A of betacoronaviruses) and SARS-CoV in the second step. PCR products were recognized in agarose gel electrophoresis and the HCoV types were finally determined by sequencing.

For the other viruses studied, RT-PCR was used for rotaviruses and caliciviruses [26,27], PCR was used for human bocaviruses [28] and ProSpecT enzyme immunoassay kits (Oxoid, Basingstoke, UK) were used for adenoviruses and astroviruses. In addition, samples that tested positive for the adenovirus antigen were also tested using the PCR method based on Allard et al. [29] to distinguish enteric adenoviruses from non-enteric types.

IBM SPSS Statistics 20 (IBM Corp., Armonk, USA) was utilized for statistical analysis. Fisher's exact test or χ^2 test were used according to the criteria for the tests, and p values below 0.05 were considered statistically significant.

3. Results

Of the 955 children studied, 595 (62.3%) were male. The median age was 14 months, with a range from 6 days to 15 years; the age distribution is shown in Table 1.

HCoVs OC43, HKU1, 229E and NL63 were detected in 19 stool samples (2.0%) and 50 nasal swab samples (5.2%). As expected, no SARS-CoV or SARS-like CoV was detected. In all but two cases of an HCoV RNA-positive stool sample, the same coronavirus was concomitantly detected in the nasal swab sample. HCoV RNA was not detected in any of the 20 available serum samples from the HCoV-positive children.

The seasonality of the HCoV findings is shown in Fig. 1. HCoV 229E and HKU1 circulated mainly during the first season, from September 2009 to August 2010, and OC43 and NL63 during the second season, from September 2010 to August 2011.

HCoV OC43 was detected in the ARTI and AGE/ARTI groups but not in the AGE group (difference between the groups was statistically significant, $p = 0.036$; Table 2), whereas HKU1, 229E and NL63 were all found in each of the three study groups, with no significant differences between the AGE, ARTI and AGE/ARTI groups ($p = 0.486$ for HKU1, $p = 0.079$ for 229E and $p = 0.362$ for NL63; Table 2). The most commonly detected virus was HCoV NL63 (15 cases), followed by OC43 (13 cases), 229E (13 cases) and HKU1 (11 cases). HCoV NL63 was detected concomitantly in stool and nasal swab samples in seven (47%) of the positive cases, and OC43 in six (46%) of the positive cases. HKU1 and 229E were mainly detected in the nasal swab samples only.

HCoVs were detected in eight (4.7%) stool samples of children in the AGE group, and in six of the eight cases, both stool and nasal

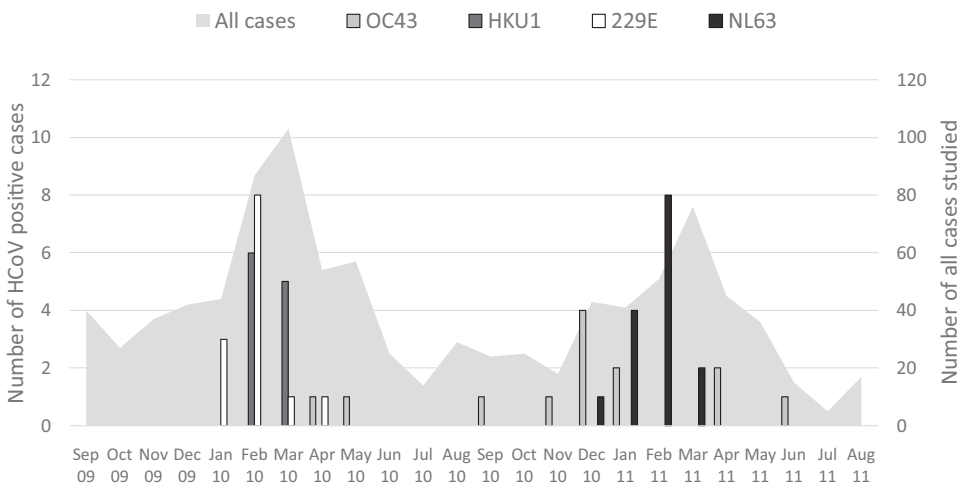

Fig. 1. Monthly distribution of human coronavirus (HCoV) positive cases and all cases studied.

Table 2
HCoV findings in the different study groups.

	AGE[a]	ARTI[b]	AGE/ARTI[c]	*p*-value[d]
Stool samples				
HCoV-OC43	0	2(0.4%)	4(1.7%)	0.079
HCoV-HKU1	1(0.6%)	1(0.2%)	0	0.390
HCoV-229E	3(1.7%)	0	0	0.006
HCoV-NL63	4(2.3%)	2(0.4%)	2(0.8%)	0.036
Nasal swab samples				
HCoV-OC43	0	6(1.1%)	7(2.9%)	0.036
HCoV-HKU1	2(1.2%)	8(1.5%)	1(0.4%)	0.486
HCoV-229E	4(2.3%)	4(0.7%)	4(1.7%)	0.183
HCoV-NL63	3(1.7%)	6(1.1%)	5(2.1%)	0.425
Combined results[e]				
HCoV-OC43	0	6(1.1%)	7(2.9%)	0.036
HCoV-HKU1	2(1.2%)	8(1.5%)	1(0.4%)	0.486
HCoV-229E	5(2.9%)	4(0.7%)	4(1.7%)	0.079
HCoV-NL63	4(2.3%)	6(1.1%)	5(2.1%)	0.362

[a] Acute gastroenteritis.
[b] Acute respiratory tract infection.
[c] Symptoms of both AGE and ARTI.
[d] Fisher's exact test.
[e] Positive stool and/or nasal swab sample.

swab samples were positive for the same HCoV. Additionally, in six of the eight cases another virus, rotavirus (three cases) or calicivirus (three cases), was also present in the same stool sample. In one child, an HCoV (HCoV 229E) was detected in the stool sample without other viruses and without a positive nasal swab sample. This 2-year-old boy was hospitalized overnight and received intravenous rehydration.

In the ARTI group, HCoV RNA was found in 24 children (4.4%); in 19 cases, it was only found in the nasal swab and in five cases, it was concomitantly in stool sample and nasal swab.

In the AGE/ARTI group, 17 children (7.1%) were positive for HCoVs. Both the stool and nasal swab samples were positive in six children, and the nasal swab was positive alone in 11 children. In five children with a positive stool sample, a known gastroenteritis virus (calicivirus in three cases, astrovirus in one and both in one) was detected in the same sample.

4. Discussion

The existence of human enteric coronaviruses has been proposed since the early EM findings of coronavirus-like particles in the stools of children with acute diarrhea, even though it was difficult to culture these coronaviruses from stools [18]. With the availability of RT-PCR methods, HCoVs have been detected in stools by the authors and others [23,24]. In our previous study, we found HCoVs OC43, 229E, NL63 and HKU1 in children with AGE, but some of the control children without gastrointestinal symptoms also harbored HCoVs in their stools [23]. The present prospective study was designed to expand on the previous one and to compare the simultaneous presence of HCoVs in stools and the respiratory tract.

All four commonly circulating HCoVs were detected in this study. With the exception of HCoV OC43, which was not detected in children with AGE only, all HCoVs were detected without significant differences in the three study groups, and all were more commonly detected in nasal swab samples than in stool samples. Therefore, none of the four coronaviruses could be specifically associated with AGE. Moreover, when HCoV was found in stools, it was almost always found concomitantly in the nasal swab too. These findings could mean that the detection in stools reflects the presence of HCoV in the respiratory tract and the virus detected in stools may only be there as the result of being swallowed. In the present study, in almost all cases of HCoV detection in both stool and nasal swab samples, the nasal swab samples were positive in the first PCR step. This suggests a relatively large quantity of viruses in the respiratory tract, compared to cases with a positive nasal swab sample only; in these cases, over half of the samples were positive only in the second PCR step. This could mean that higher virus load in the respiratory tract results in the presence of the virus in the stool either due to swallowing or by other mechanism. In most of the HCoV-positive stool samples, only the second PCR step was positive, indicating a relatively small quantity of viruses.

However, it has been shown that SARS-CoV can be isolated by culturing intestinal tissue samples and the intestinal biopsy specimens showed the presence of active viral replication in the intestines of patients with SARS [21], so the presence of SARS-CoV RNA in the stool is not only a consequence of the passive shedding of the virus from the respiratory tract. This is of interest at least for HCoV NL63, which uses the same ACE-2 receptor as SARS-CoV for cellular entry [30].

None of the sera studied was positive for HCoV RNA. These findings suggest that viremia is not common in HCoV infections, supporting the results of a Slovenian study in which whole blood samples of all HCoV-positive children (on the basis of a stool or nasopharyngeal sample) were negative [24].

To conclude, the four commonly circulating human coronaviruses, representing alphacoronaviruses and lineage A of the betacoronaviruses, can occasionally be found in stool samples of

children with AGE, but in almost all cases the virus is simultaneously detected in the respiratory tract, which is likely the source of the virus. HCoV findings in stools are more likely to be derived from respiratory infection, and the gastroenteritis symptoms can be explained by the well-known gastroenteritis viruses found in the same stool samples. Therefore, it seems that commonly circulating HCoVs do not have a significant role in the etiology of gastrointestinal infections in hospitalized children.

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Competing interest

None declared.

Ethical approval

The study was approved by the Ethics Committee of Pirkanmaa Hospital District (ETL code: R09070).

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REGULAR ARTICLE

Human bocavirus types 1, 2 and 3 in acute gastroenteritis of childhood

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ABSTRACT

Aim: Recently identified human bocavirus (HBoV) types 2 and 3 have been associated with acute gastroenteritis in children. We studied 878 stool specimens from children with acute gastroenteritis and 112 controls (43 children with unspecified fever, 33 with respiratory tract infection and 36 healthy children) for known HBoVs. The same specimens were previously studied for rotaviruses, noroviruses, sapoviruses, adenoviruses, coronaviruses and aichivirus.

Methods: HBoVs were detected by PCR and positive amplicons were sequenced to identify HBoV1, HBoV2, HBoV3 and HBoV4.

Results: HBoV of any type was found in 85 (9.7%) cases of acute gastroenteritis and in 6 (5.4%) controls. HBoV1 was detected in 49 (5.6%) cases and 2 (1.8%) controls, HBoV2 in 29 (3.3%) cases and 2 (1.8%) controls and HBoV3 in 8 (0.9%) cases and 2 (1.8%) controls. No HBoV4 was found. HBoV as a single infection was found in 16 (1.8%) cases and in 6 (5.4%) controls; in the remaining cases, a known gastroenteritis virus was also found. Among the single HBoV infections, HBoV2 was the most common type with 8 (50%) cases.

Conclusion: HBoVs are rarely found alone in children with acute gastroenteritis. Further studies are warranted to confirm a possible specific association of HBoV2 with gastroenteritis.

INTRODUCTION

The first human bocavirus (HBoV1) was described in 2005 by Allander et al. (1) as a result of screening of nasopharyngeal aspirates of children with respiratory tract infection. Since then, HBoV1 has been connected to respiratory tract infections (2–4), and the evidence for this has increased with the use of serological studies (5). On the other hand, co-infections with other respiratory pathogens are common and HBoV1 has also been found in asymptomatic subjects (6–8).

Furthermore, several studies have shown that HBoV1 may also be present in faecal samples of children with acute gastroenteritis (AGE) (9–13), and to complicate matters, patients with HBoV1 in respiratory tract samples have been reported to have diarrhoea (3). As in the case of the respiratory tract, simultaneous presence of HBoV1 with other, previously established gastroenteritis viruses is common in faecal specimens (10,12,13), and no clear connection between HBoV1 and AGE of children has been established (11,13,14).

Since 2009, three new human bocaviruses have been identified (14–16). HBoV2 was found in a stool specimen of a Pakistani child in 2009 (15), and since then, HBoV2 has been detected in several studies on children with AGE (14,17–21). Again, co-infections with known human gastroenteritis viruses have commonly been found (18,20) as well as shedding in stools of asymptomatic children (20). Therefore, the role of HBoV2 as an enteric pathogen is still not confirmed. HBoV2 has also been detected in stools of

Abbreviations
AGE, acute gastroenteritis; HBoV, human bocavirus; HBoV1, human bocavirus 1; HBoV2, human bocavirus 2; HBoV3, human bocavirus 3; HBoV4, human bocavirus 4; PCR, polymerase chain reaction

Key notes

- We found human bocaviruses (HBoVs) 1, 2 and 3 at respective rates 5.6%, 3.3% and 0.9% in children with acute gastroenteritis but mostly in combination with known gastroenteritis viruses.
- Of the cases with HBoV as a single agent (1.8% of all AGE cases), HBoV2 accounted for 50%.
- Further studies are warranted to confirm the possible role of HBoV2 in acute gastroenteritis; otherwise, the role of human bocaviruses appears small.

children with respiratory tract infection (22) but not at all (23) or rarely (24) in respiratory tract samples.

HBoV3 was also originally detected in a stool sample in 2009 (14). Several other studies have confirmed the presence of HBoV3 in faecal samples of patients with gastroenteritis, but detection rates have been lower than those of HBoV2 (19–21).

Recently, HBoV4 was found in faecal samples of children and adults (16), but the significance of this virus may be regarded as unknown.

To elucidate the role of different human bocaviruses in AGE of children, we tested stool specimens from 878 children seen in hospital because of AGE. The samples were collected in a 2-year prospective study from August 2006 to August 2008 (25). This material had been tested previously for known gastroenteritis viruses including rotaviruses, noroviruses, sapoviruses, adenoviruses, coronaviruses and aichivirus (25–29).

MATERIAL AND METHODS

Patients and specimens

The prospective study on the aetiology of AGE in children was approved by the Ethics Committee of Pirkanmaa Hospital District and conducted at Tampere University Hospital from August 2006 to August 2008 and at Kuopio University Hospital from September 2006 to August 2007 (25).

Children under 15 years of age with AGE admitted to the paediatric outpatient clinic or to the hospital ward, or those who came down with AGE during hospitalization were eligible for the study. Informed consent was obtained from the guardians of all study subjects diagnosed with AGE by a paediatrician or included in the study as controls. A total of 878 stool specimens were obtained from children with AGE (one per subject) and 112 stool specimens were collected as controls including three different groups of patients: 43 specimens from children with fever of unknown origin (some of these also had vomiting but not diarrhoea), 33 specimens from children with respiratory tract infection and 36 specimens from healthy children admitted for examinations. Because the study material was not originally collected for HBoV studies, the selection of the control material was not optimal for HBoVs. Also, the number of control cases remained small causing some limitations for statistical analyses, as discussed later.

Rotaviruses, caliciviruses including norovirus genogroups I and II and sapoviruses, and human coronaviruses (229E, OC43, HKU1 and NL63) had previously been studied using the same material (25–27). In addition, 516 stool specimens were previously tested for aichivirus and 724 for adenoviruses (28,29). All enteric viruses were tested using polymerase chain reaction (PCR) method, except for adenoviruses; either a PCR or ProSpecT® enzyme-linked immunosorbent assay-kit (Oxoid, Basingstoke, UK) was used.

Laboratory methods

Suspensions 10% w/v were made by diluting stool specimens in phosphate-buffered saline. Viral nucleic acid was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol (this method was tested to be suitable for DNA extraction).

HBoV DNA was amplified by PCR, and a two-step PCR method was used to increase sensitivity. In the first PCR, reaction volume was 50 μL containing 5 μL of the sample DNA, 1× Green GoTaq® Flexi Buffer (Promega, Madison, WI, USA), 1.5 mmol/L of GoTaq® $MgCl_2$ (Promega), 200 μmol/L of each dNTP (Promega), 2.5 U of GoTaq DNA polymerase (Promega) and 0.5 μmol/L of HBoV NS1 primers (Sigma-Aldrich, St Louis, Mo, USA). PCR programme was run as follows: denaturation at 94°C for 3 min, 35 cycles of amplification (40 sec at 94°C, 30 sec at 62°C, 65 sec at 72°C) and final extension at 72°C for 5 min. The first amplification produced a 960-bp amplicon of gene NS1 encoding for a non-structural protein (Table 1).

In the second PCR, there were two pools of primers, pool Boca (30) and pool HBoV, producing amplicons of 291 and 200 bp in size, respectively (Table 1). Pool HBoV-primers were designed to detect all HBoVs, especially the newer ones, and pool Boca primers already in use in our laboratory for HBoV1 were included in the PCR. Reaction volume was 50 μL containing 2 μL of the 1st PCR product, 150 μmol/L of each dNTP and 0.5 μmol/L of HBoV NS1 2nd primers or Boca NS-1 primers, and the rest of the reaction conditions were as in the 1st PCR. PCR programme was run as follows: 3 min at 94°C, 30 cycles of amplification (30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C) and at 72°C for 5 min.

Table 1 The oligonucleotide primers for HBoVs

Name	Sequence*	Position**	Size
HBoV NS1 fwd	GGACGTGGT**S**CGTGGGAAC	1089–1107(+)	960 bp
HBoV NS1 rev	GTCCTGTGAATG**W**GTAGGACAAAGG	2024–2048(−)	
HBoV NS1 2nd fwd	CC**W**GTAATTAT**W**TCCACTAACCA	1764–1786(+)	200 bp
HBoV NS1 2nd rev	AGAGTACA**K**TCGTACTCATT**R**AA	1941–1963(−)	
Boca NS-1 fwd***	TATGGCCAAGGCAATCGTCCAAG	1545–1567(+)	291 bp
Boca NS-1 rev***	GCCGCGTGAACATGAGAAACAGA	1813–1835(−)	

*IUB (the International Union of Biochemistry) codes in bold: S = G or C, W = A or T, K = G or T, R = A or G.
**In genome of HBoV2 isolate KU1 (GQ200737).
***By Sloots et al. (30).

PCR products were visualized in gel electrophoresis, and positive results were confirmed by sequencing using ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were analysed using Sequencher™ 4.8 program (Gene Codes Corporation, Ann Arbor, MI, USA) and compared to reference strains by NCBI Blast®-program to determine HBoV types.

Statistical methods

Statistical analyses were conducted using PASW Statistics 18 program (SPSS®, Chicago, IL, USA). Statistical significance was calculated using Fisher's exact test, and $p < 0.05$ was considered significant.

RESULTS

Altogether, 878 stool specimens were collected from children with AGE and 112 specimens from control groups including 43 specimens from children with fever of unknown origin, some of whom also had vomiting, 33 specimens from children with respiratory tract infection, and 36 specimens from healthy children admitted for examinations. In 719 (81.9%) of the 878 stool specimens of children with AGE, one or more viruses, including rotaviruses, noroviruses, sapoviruses, adenoviruses, coronaviruses, aichivirus and human bocaviruses, were found. In the combined control groups, 22 (19.6%) of 112 specimens were positive for at least one of these viruses, excluding sapoviruses and aichivirus that were not detected from controls.

Human bocaviruses were detected in 91 (9.2%) cases of all stool samples, 85 (9.7%) of the 878 cases with AGE and 6 (5.4%) of the 112 (non-AGE) controls. There was not a statistically significant difference in the amount of HBoV-positive cases between AGE group and combined controls ($p = 0.165$).

HBoV1 was detected in 49 (5.6%) cases of AGE. There was also one (2.3%) positive sample in the group of children with fever of unknown origin and one (3.0%) positive sample in the group of children with respiratory tract infection; in the healthy children, there were no positive samples. (Table 2.) The differences in detection rates of HBoV1 between AGE and the separate control groups were not statistically significant ($p = 0.529$). During the first season (August 2006–August 2007), there were 20 cases positive for HBoV1 and during the second season (September 2007–August 2008), 31. One of the samples in the AGE group contained both HBoV1 and HBoV2.

Twenty-nine (3.3%) specimens in the AGE group were positive for HBoV2. In the control groups, there was one (2.3%) positive sample in children with fever of unknown origin and one (2.8%) positive sample in the group of healthy children, but none in the children with respiratory tract infection. (Table 2.) The differences in detection rates of HBoV2 between AGE and the separate control groups were not statistically significant ($p = 0.949$). Numbers of HBoV2-positive samples during the first and the second season were 18 and 13, respectively.

Table 2 Number and per cent of human bocavirus (HBoV)-positive cases among different study groups

	Number (%) of stool specimens in different study groups				
Virus	AGE* (N = 878)	Fever** (N = 43)	RTI*** (N = 33)	Healthy children (N = 36)	All (N = 990)
HBoV1	49 (5.6%)	1 (2.3%)	1 (3.0%)	0	51 (5.2%)
HBoV2	29 (3.3%)	1 (2.3%)	0	1 (2.8%)	31 (3.1%)
HBoV3	8 (0.9%)	1 (2.3%)	1 (3.0%)	0	10 (1.0%)
Total	86 (9.8%)†	3 (7.0%)	2 (6.1%)	1 (2.8%)	92 (9.3%)†

*Children with acute gastroenteritis.
**Children with unknown fever.
***Children with respiratory tract infection.
†One of the samples contained both HBoV1 and HBoV2, so this case is calculated here twice. The actual number of HBoV-positive cases in AGE group is 85 (9.7)% and in all samples 91 (9.2)%.

HBoV3 was detected in 8 (0.9%) cases of children with AGE, in one (2.3%) case of children with fever of unknown origin, and in one (3.0%) case of children with respiratory tract infection, and in none of the healthy children (Table 2.). The differences in detection rates of HBoV3 between AGE and the separate control groups were not statistically significant ($p = 0.260$). All HBoV3-positive cases were found during the first season.

In this study, no HBoV4 was detected.

In 16 (1.8%) cases of AGE, human bocavirus was the only virus detected, whereas in the combined controls, all 6 (5.4% of all controls) cases were single infections with HBoV. Among the 16 cases of AGE in which HBoV was the only virus detected in stool, HBoV2 was the most common one with 8 (50.0% of all single infections) cases, followed by HBoV1 with 7 (43.8%) cases and HBoV3 with one (6.3%) case (Table 3). Conversely, among the 69 cases of mixed infections, 42 (60.9% of the HBoV-positive mixed infections) contained HBoV1, 21 (30.4%) contained HBoV2, and 7 (10.1%) cases contained HBoV3. In one sample, both HBoV1 and HBoV2 were detected. The proportion of HBoV2 was greater in the single infections than in mixed infections and vice versa for HBoV1, but the differences in the proportions of different HBoVs between mixed and single infections were not statistically significant ($p = 0.428$).

Table 3 Human bocavirus (HBoV)-positive findings with other viruses in acute gastroenteritis of childhood

		Co-infection with one virus					
HBoV	HBoV alone	*Rota*	*Noro*	*Sapo*	*Adeno*	Co-infection with more than one virus*	Total
HBoV1	7	14	13	0	5	10	49
HBoV2	8	11	5	1	1	3	29
HBoV3	1	4	2	0	0	1	8
Total	16	29	20	1	6	14**	86**

*Including rotaviruses (8), noroviruses (4), sapoviruses (2), adenoviruses (1), aichivirus (4), coronaviruses (3).
**The case including both HBoV1 and HBoV2 calculated twice.

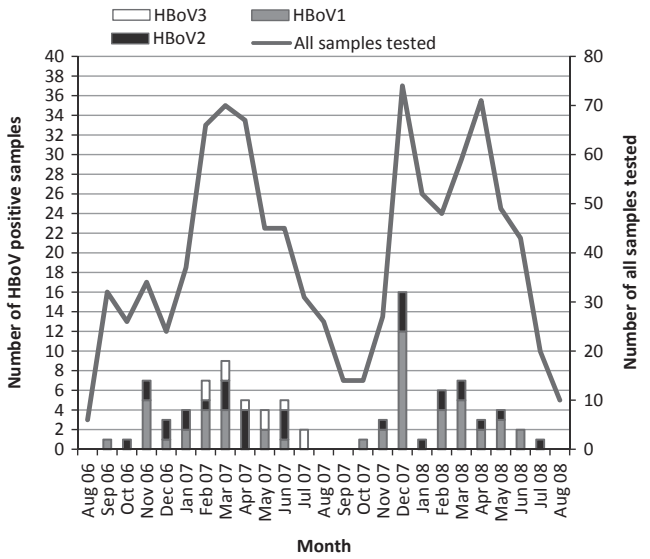

Figure 1 All human bocavirus (HBoV)-positive findings and monthly numbers of all tested samples during the study period, August 2006–August 2008. (Notice two vertical scales).

Forty-two (85.7%) of 49 HBoV1-positive cases, 21 (72.4%) of 29 HBoV2-positive cases and 7 (87.5%) of 8 HBoV3-positive cases were mixed infections with known gastroenteritis viruses. Proportion of mixed infections did not differ significantly between different HBoVs (data not shown).

In mixed infections, rotaviruses and noroviruses were the most common gastroenteritis viruses detected with bocaviruses (Table 3.). In six cases negative for rotaviruses and noroviruses, HBoVs were detected together with adenovirus. Fifty-six of the mixed infections contained two different viruses, and in 13 cases, there were more than two viruses. The one case with both HBoV1 and HBoV2 in the stool also had rotavirus in the same specimen.

The seasonal distribution of HBoV findings is shown in Fig. 1. Most of the HBoV-positive cases were detected from November to June. From November to March, over 10% of all stool samples collected per month were positive for some of the HBoVs, exception being January 2008 with low detection rate of 1.9%.

DISCUSSION

This study was set-up to examine whether HBoVs in general and bocavirus types HBoV2 and HBoV3 in particular could be linked with AGE in childhood as aetiological agents. As a whole, we could not confirm such an aetiological role of any of the HBoVs in AGE in children, although HBoV2 appeared more common than the other types when HBoV was found in stool without other viruses.

In this study, human bocaviruses were detected in 9.7% of children with AGE. HBoV1 was detected in 5.6% of all AGE cases, which concurs with previous studies of HBoV1 in children with AGE (0.8–9.1%) (10,12–14). However, the differences in the rate of HBoV1 in AGE and control groups were not statistically significant, and in very few cases, HBoV1 was detected alone without well-established gastroenteritis viruses such as rotavirus or norovirus, which were likely to have a causative role in such cases. As a single infection, HBoV1 was found in only 0.8% of AGE cases.

As shown in Fig. 1, there is a peak in HBoV1 findings during December 2007. This peak is most likely due to the waterborne AGE outbreak that occurred in Nokia, a town near to Tampere, and was caused by contamination of drinking water by sewage water (29). During the outbreak, there was an unusual amount of mixed infections with severe gastrointestinal symptoms (29). Fifty stool samples from children connected to the Nokia outbreak were tested during our study, and we found eight HBoV1-positive cases (all of these were mixed infections) and two HBoV2-positive cases (one was a mixed infection). Even if all 'Nokia cases' were excluded from the calculations, the differences between HBoVs (separately for HBoV1, HBoV2 and HBoV3) in AGE and control groups did not change outstandingly.

HBoV2 was detected in 3.3% of the samples of the children with AGE. This is a lower detection rate than those reported from Australia (17.2%) (14) and China (20.4–24.6%) (17,20), but close to the 3.6% reported from South Korea (18). HBoV2 was detected as a single virus in 0.9% of AGE cases. While we could not confirm a specific association of HBoV2 with AGE in children, there were nevertheless numerically more 'pure' cases (eight in all) of HBoV2 than other HBoVs, leaving a small possibility of a specific association of HBoV2 with gastroenteritis. Arthur et al. (14) in their case–control study found a statistically significant association between HBoV2 and AGE, but in other studies, despite the common findings in stool specimens, the causal association of HBoV2 and AGE has been weak (17,20).

In this study, HBoV3 was found in 0.9% of the AGE samples over 2 years. HBoV3 was not detected during the second season at all, and there was only one case of HBoV3 as a single virus in specimen without other gastroenteritis viruses. In earlier studies, detection rates of HBoV3 in children with AGE have been 0.9–2.7% (14,20), and in general, HBoV3 has been less common than HBoV2 in stool samples of children with AGE (19,20).

We were unable to find any HBoV4 in our study. This was also the case in several other recent studies (19–21), and therefore, the role of this virus remains unclear.

We did a thorough work-up of most of the established gastroenteritis viruses including rotaviruses, noroviruses, sapoviruses, enteric adenoviruses, coronaviruses and aichivirus (astroviruses or bacterial pathogens were not studied) and found co-infections in 81.2% of all bocavirus-positive AGE cases. Similar rates (approximately 74–80%) have been detected in earlier studies in which other gastroenteritis viruses have been investigated using adequate methods (20,21). In such co-infections, it is reasonable to assume that the known gastroenteritis viruses actually have a causative role and HBoV may be either shed from the respiratory tract or infecting the intestinal tract with no pathogenic role

in AGE. In the future, simultaneous testing of respiratory and stool samples together with serologic testing should be carried out to clarify this assumption.

The number of the 'pure' HBoV-positive cases was small and could not be positively associated with AGE. Nevertheless, it is noteworthy that in 50.0% of the single infections, HBoV2 was the bocavirus detected, whereas in the mixed infections, its proportion was only 30.4%.

In this study, most of the HBoV-positive cases were detected from November to June. The highest proportional detections rates, comparing to number of collected samples per month, were in winter months (Fig. 1). There was no remarkable difference in seasonality between HBoV1 and HBoV2, but HBoV3 was detected only from February 2007 to July 2007. In some previous studies, HBoV1 was detected throughout the year, but some higher incidence during winter months was also seen (3,11). HBoV2 was also detected throughout the year, and the highest incidences were detected from February to April (17).

The sizes of the control groups were a limitation in our study and also a limitation for a reliable statistical analysis of causative role of HBoVs in AGE. In principle, using all three groups, that is, children with respiratory tract infection, children with fever and vomiting, and healthy children as controls, might be justified, but the size of each group remained too small, and some statistical comparison was made with pooled controls, which is not optimal. Originally, this material was not collected for bocavirus studies. Because of these limitations and because other viruses were frequently found in cases of AGE, we focused more on infections with HBoV as a single pathogen indicating specific association with AGE.

Further studies are warranted, and a study with simultaneous collection of specimens from respiratory tract and stools is in progress to investigate the type-specific association of HBoVs with respiratory or gastrointestinal tract, respectively. Collection of the serum samples is also being carried out in our next studies for serological testing and the detection of HBoV viraemia.

In conclusion, we investigated stool samples from a large number of children with AGE and found human bocaviruses 1, 2 and 3 at respective rates of 5.6%, 3.3% and 0.9%, but bocaviruses were seldom detected alone without other viruses like rotaviruses and noroviruses. Even those cases that appeared to be single HBoV infections may actually have been co-infections if a more comprehensive work-up on viruses such as astroviruses had been performed and bacterial pathogens had also been investigated. In the total material, HBoV2 and HBoV3 did not stand out as having any stronger association with AGE than HBoV1, but in the 'pure' HBoV cases of AGE, HBoV2 was slightly overrepresented.

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CONFLICT OF INTEREST AND FUNDING

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Human bocaviruses are commonly found in stools of hospitalized children without causal association to acute gastroenteritis

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Authors` summary

Human bocaviruses (HBoVs) 1-4 have been found in stool samples of children, but without clear association to acute gastroenteritis (AGE).

We collected a large material of stool samples and nasal swab samples from children with AGE and various diagnoses of acute respiratory tract infection (ARTI) to study the connection between HBoVs and AGE.

We found out that HBoV1 was commonly found in stool samples and nasal swab samples, but finding of it in stool was a sign of respiratory infection rather than gastrointestinal infection.

HBoV2 was common in stool samples of children, but was found equally in children with AGE, ARTI or symptoms of both. In only one case with AGE HBoV2 was detected alone in stool, in other cases there were other viruses like rotavirus or norovirus detected in the same stool sample and were probably causing the symptoms.

HBoV3 was quite rare finding, and couldn´t be connected to AGE either. HBoV4 was not found.

In conclusion, HBoV2 and HBoV3 were more commonly found in stool than nasal swab samples, but the findings could not be causally linked with AGE. HBoV1 was commonly found in stool samples during ARTI, with or without gastrointestinal symptoms.

Abstract

Human bocaviruses (HBoVs) may be grouped into respiratory (HBoV1) and enteric (HBoV2–4) types. We examined this association of HBoV types and clinical symptoms in 955 children who had acute gastroenteritis (AGE, n=172), acute respiratory tract infection (ARTI, n=545) or symptoms of both (n=238). Both nasal swab and stool specimens were studied for such patients.

HBoV1 DNA was detected in 6.2% of patients with ARTI and 9.2% of patients with symptoms of both ARTI and AGE, but in only 1.7% of patients with AGE alone. In about one-half of the cases HBoV1 was detected concomitantly in nasal swab and stool samples. HBoV2 was found in stool samples of patients with AGE (5.8%), ARTI (5.1%) and symptoms of both (5.5%) but only rarely in nasal swabs. HBoV3 was found in the stools, but not in nasal swabs, in 0.6%, 1.1% and 0.8% of patients with, respectively, AGE, ARTI and both. HBoV4 was not found. All but one HBoV -positive stool samples of AGE patients contained a known gastroenteritis virus (rotavirus, norovirus, sapovirus, astrovirus or enteric adenovirus) that was probably responsible for the symptoms of the respective case. Sera of 30 HBoV-positive patients were available and IgM antibodies for HBoVs were found in 10 cases and HBoV DNA in eight of these.

Conclusions: HBoV2 and HBoV3 were more commonly found in stool than in nasal swab samples, but the findings could not be causally linked with AGE. HBoV1 was commonly found in stool samples during ARTI, with or without gastrointestinal symptoms.

Key words: human bocavirus, acute gastroenteritis, acute respiratory tract infection, children

Abbreviations: acute gastroenteritis (AGE), acute respiratory tract infection (ARTI), human bocavirus (HBoV)

Introduction

There are four recognized types of human bocavirus (HBoV), all of which may be associated with disease in children. HBoV1 was detected in nasopharyngeal aspirates of children in 2005 [2] and is recognized as a respiratory pathogen in children [1, 9, 19, 22, 23, 28]. While HBoV1 has also been found in stools of children with diarrhoea, there may not be a causal association with acute gastroenteritis (AGE) [8, 24]. The presence of HBoV1 in stools may be long lasting and asymptomatic [18].

HBoV2 was discovered in 2009 in stool samples [17]. One study found a significant association between HBoV2 and gastroenteritis in children [5], but in other studies the association has been weaker [12, 24, 26]. HBoV2 has only occasionally been found in the respiratory tract [10, 21, 27].

HBoV3 was likewise discovered in stool samples in 2009 [5]. The detection rates have been lower than those of HBoV2, and no association between HBoV3 and AGE has been confirmed [5, 14, 16, 24, 26, 29]. HBoV3, too, has been found only infrequently in respiratory tract samples [21].

The fourth member of this group, HBoV4, was discovered in stools in 2010 [16], but so far the findings of HBoV4 have been scarce in both stool and respiratory tract samples [16, 20, 21].

This prospective study was set up to further elucidate the association of various HBoV types with clinical entities in children. We specifically wanted to examine the proposed causal association between HBoV2 and AGE.

Materials and methods

Patients and samples

This study was conducted from September 2009 to August 2011 in the Department of Paediatrics at the Tampere University Hospital. The study was approved by the Ethics Committee of Pirkanmaa Hospital District and informed consent was obtained from the parents of all the children enrolled.

Children with AGE who were seen as outpatients or admitted as inpatients were eligible for the study. To limit the number of recruited subjects for acute respiratory tract infection (ARTI), only children admitted as inpatients were eligible. A study nurse or delegated paediatric nurses recruited eligible children when they were seen in the paediatric outpatient clinic or after they were admitted to the hospital ward. The

diagnoses of AGE or ARTI were made by paediatricians (consultants or residents) in the hospital. If the diagnosis code was unspecific (e.g. fever) but symptoms indicated AGE or ARTI, the patient's participation and study group were confirmed afterwards by the investigator; in these cases, decisions were based on the medical records. If a child was admitted to the hospital more than once during the study period, the admissions were considered to represent two separate episodes if the child had been asymptomatic for at least 14 days between the admissions. Stool samples and nasal swab samples were intended to be collected from all participants. A blood sample was obtained for the study only if one was taken for diagnostic or treatment-related reasons.

The patients were retrospectively divided into three groups: the AGE group (patients with gastrointestinal symptoms only), the ARTI group (patients with symptoms of respiratory tract infection only), and the AGE/ARTI group (patients with different combinations of the symptoms of both AGE and ARTI).

Altogether, 1610 children were recruited into the study. In 955 cases, both stool and nasal swab samples were obtained and studied for HBoVs. The 955 cases included 172 children with AGE, 545 with ARTI and 238 with symptoms of both. Of these children, 595 (62.3%) were male. The median age was 14 months: 224 of the study patients (23.5%) were less than six months old, 481 (50.4%) were 6–24 months old, 178 (18.6%) were 2–5 years old and 72 (7.5%) were over five years old. One hundred and thirty-seven patients (14.3%) were treated as outpatients and 818 (85.7%) as inpatients; the median length of the hospital stay was two days.

In the AGE and AGE/ARTI groups, stool specimens were also studied for rotaviruses, noroviruses and sapoviruses using RT-PCR methods [11, 25]. In addition, HBoV-positive stools were tested for adenoviruses and astroviruses using ProSpecT enzyme immunoassay kits (Oxoid, Basingstoke, UK). Samples positive for adenovirus antigen were also studied using PCR (method based on Allard et al.[3]) to distinguish enteric adenoviruses from other adenovirus types.

Laboratory methods

Stool specimens were diluted in phosphate-buffered saline to prepare 10% suspensions. Nasal swab specimens were collected in UTM-RT Mini tubes (Copan Italia, Brescia, Italy), mixed, centrifuged and used for extraction.

Viral nucleic acid was extracted from stool suspensions and nasal swabs using QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol (this method was tested and shown also to be suitable for DNA extraction). For serum samples, the QIAamp DNA Mini Kit (QIAGEN) was used for extraction.

HBoV DNA from stool and nasal swab samples was amplified using a qualitative PCR method [26]. Primers amplifying all HBoV types were targeted to the gene encoding the non-structural protein NS1. PCR products were visualized in gel electrophoresis, and positive results were sequenced using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequences were analysed using the Sequencher 4.9 program (Gene Codes Corporation, Ann Arbor, USA) and compared to the reference strains using BLAST (US National Library of Medicine, Bethesda, USA) to determine the HBoV types.

HBoV1-4 DNA from serum samples was detected using quantitative PCR with HBoV-type-specific primers covering the left-hand untranslated region of the HBoV genome and the beginning of the NS1 gene [14]. HBoV1–3-specific IgM and IgG antibodies were detected in sera using virus like particle - competition enzyme immunoassays as described earlier [13].

Statistical methods

The IBM SPSS Statistics 20 program (IBM Corp., Armonk, USA) was used for statistical analysis. The χ^2 test and Fisher's exact test were used as appropriate, and p-values below 0.05 were considered statistically significant.

Results

HBoV1 DNA was detected in 59 (6.2%) of 955 cases, HBoV2 DNA in 51 (5.3%), HBoV3 DNA in nine cases (0.9%) and HBoV4 in none. The seasonal distributions of all the cases studied and HBoV DNA-positive cases are shown in **Figure 1**. The age distribution of HBoV1 and HBoV2 DNA-positive findings is shown in **Figure 2**. Proportionally, most positive findings were in children 6–24 months of age.

HBoV1 in stool and nasal swab samples

In the AGE group, HBoV1 DNA was detected in only three patients (1.7%). In one child, HBoV1 DNA was found in both stool and nasal swab samples, in another child in stool and in a third child in a nasal swab sample only. In the latter, other viruses were not detected in stool samples, but in the first two, norovirus (one case) or sapovirus (one case) was detected.

In the ARTI group, HBoV1 DNA was detected in 34 patients (6.2%) from at least one sample type. HBoV1 was found simultaneously in the stools and nasal swabs in 18 patients (52.9% of all 34 positive cases). In one of the children, HBoV1 DNA was detected in the nasal swab, but the stool sample was positive for HBoV3 DNA.

In the AGE/ARTI group, HBoV1 DNA was detected in 22 patients (9.2%): in 12 children (54.5% of all 22 positive cases) concomitantly in the stools and nasal swabs. In one of the children HBoV1 DNA was detected in the nasal swab, but the stool sample was positive for HBoV2 DNA.

The difference in the proportion of HBoV1-positive children between the study groups was statistically significant ($p= 0.008$) (Table 1): in other words, HBoV1 was found significantly more often when respiratory symptoms were present compared with gastroenteritis symptoms only.

HBoV2 in stool and nasal swab samples

In the AGE group, HBoV2 DNA was detected in ten patients (5.8%), all of whom had HBoV2 DNA in stools, and one in the nasal swab as well. Only once was HBoV2 the sole virus detected in the stool sample; the remaining nine children had also rotavirus (five cases), norovirus (three cases) or enteric adenovirus (one case) in their stool.

In the ARTI group, 28 cases (5.1%) harboured HBoV2 DNA, all in the stool samples. In only two patients was HBoV2 DNA detected in the nasal swabs as well.

In the AGE/ARTI group, HBoV2 DNA was detected in 13 stool samples (5.5%). In eight of the 13 cases, the main diagnosis was AGE and the respiratory symptoms were mild, and, in five of these children, other viruses – norovirus (three cases), enteric adenovirus (one case) or rotavirus and sapovirus (one case) – were also detected in stools.

The difference in the proportion of HBoV2-positive cases between the study groups was not statistically significant (p= 0.941; Table 1).

Because of the long study period, some of the children were recruited to the study more than once. There were five such cases among HBoV-positive children, and in three cases, HBoV (specifically HBoV2) DNA was detected in stool samples during two consecutive admissions. The time period between the two HBoV2-positive stool samples varied from three weeks to four months, and in all cases the principal diagnosis was respiratory infection.

HBoV3 in stool and nasal swab samples

HBoV3 DNA was detected only in nine stool samples; none of the nasal swab samples were positive. The difference between the three groups was not statistically significant (p= 1.000; Table 1). All HBoV3 DNA-positive cases in the AGE and the AGE/ARTI groups were co-infections with rotavirus (one case) or norovirus (two cases).

HBoV antibodies and DNA in serum

Thirty acute-phase sera and five convalescent-phase sera (taken two to four weeks after the first serum sample) of 117 patients with HBoV DNA in stools and/or nasal swabs were available for serology. HBoV IgM antibodies were found altogether in ten of 30 acute-phase sera; in two cases, IgG -seroconversions were also observed in paired sera. Eight of the ten sera were also HBoV DNA -positive with a 100% concordance in a virus type with the serodiagnoses (Table 2).

In children with HBoV1 DNA in stools or nasal swabs, IgM antibodies specific to HBoV1 were detected in six (five had also HBoV1 viremia) of 11 available serum samples in the AGE/ARTI group (Table 2), but not in the single available serum in the AGE group.In HBoV2-positive (stool or nasal swab) cases, HBoV2-specific IgM, and HBoV2 viremia, was detected in two of six available sera in the AGE group, in one of six sera in the AGE/ARTI group, but in none of four sera in the ARTI group (Table 2). IgM antibodies were not detected in three available sera of the nine HBoV3-positive patients. However, one of the cases in the AGE/ARTI group had HBoV3 DNA in serum.

Discussion

The aim of our study was to investigate the disease associations of different HBoV types, especially the alleged connection of HBoV2 with AGE in childhood. We studied the question using a clinical material consisting of AGE and various diagnoses of ARTI in children. It is a common clinical experience that symptoms of AGE and ARTI may occur simultaneously, and for this reason we formed a third study group of children with symptoms of both.

Our first finding was that HBoV1 was an uncommon finding in children with AGE, but was found commonly in both stool and nasal swab samples of patients with ARTI and those with ARTI and AGE symptoms, in line with being a respiratory tract pathogen. When HBoV1 was found in stools, other gastroenteritis viruses (rotavirus, norovirus, adenovirus or astrovirus) were usually also found, readily explaining the AGE symptoms. Rates of detection of HBoV1 in the ARTI and AGE/ARTI groups were 6.2% and 9.2% respectively, which is in line with the 5.6–19% found in other studies [1, 4, 6, 9, 21, 27]. In one-half of the HBoV1-positive cases, HBoV1 was detected simultaneously in stool and nasal swab samples. Most such cases occurred in the ARTI group. Whether these findings indicate passage of HBoV1 through the gastrointestinal tract due to swallowing of respiratory secretions, or true replication of the virus in the gastrointestinal tract cannot be concluded. In any case it appears that HBoV1 can be shed in stools without causing any gastrointestinal symptoms.

HBoV2 was detected at similar rates, around 5%, in the stools of children with AGE, ARTI and with symptoms of both, but rarely in nasal swabs. In previous studies, the detection rates have been 1.4–20.4% [5, 12, 20, 26], and mixed infections with other viruses have been commonly found [12, 26]. In the present study, only one sample harboured HBoV2 alone in the AGE group; in the other HBoV2-positive cases, the same stool sample contained rotavirus, norovirus or enteric adenovirus, all of which are known causative agents of AGE and probably responsible for the AGE symptoms in our patients. Five of the 14 HBoV2-positive stool samples from the AGE/ARTI group also contained known gastroenteritis viruses. Whether HBoV2 had any pathogenic role in these cases remains doubtful, but shedding of the virus in stools appears to be common. Long shedding of HBoV2 is also supported by the observation that, in three children, HBoV2 was detected in stool samples from two consecutive admissions, with time periods from

three weeks to four months between samples. We conclude that HBoV2 is more likely found in stool than nasal swab samples, but it appears unlikely that HBoV2 would be a causal virus of AGE in children.

The present study confirmed previous findings [14, 26] that HBoV3 is not a common virus in stool samples of Finnish children. The detection rates observed elsewhere have also been low, varying from 0.9% to 2.7% [5, 12, 20]. While the connection of HBoV3 with the gastrointestinal tract (stool specimens) was evident, the association with AGE was not. Indeed, HBoV3 showed no connection with any of the clinical groups in this study.

HBoV4 DNA was not detected in this study, nor was it found in our previous studies [14, 26] or in several studies from other countries [7, 29]. We conclude that circulation of HBoV4 in Finnish children is rare.

We did not have a healthy control group, which is a limitation of this study. It would be difficult to collect a large number of nasal swabs and stool samples from healthy children. However, we believe that the comparison of findings in large groups of patients with AGE and ARTI was sufficient to delineate the roles of HBoV1 and HBoV2 in these conditions. Another limitation might be the use of qualitative PCR for HBoVs (for stool and nasal swab samples). In previous studies, high viral loads of HBoV1 in respiratory samples have correlated with acuteness of infection and absence of other viruses [1, 6, 15, 28], but, on the other hand, there has been no significant difference in HBoV copy numbers between stool samples from patients with gastroenteritis and healthy controls [8, 12, 24].

In this study, we used HBoV IgM serology and serum PCR for HBoV DNA to define acute infection. Unfortunately, as a result of the study protocol, serum samples were available for only one-fourth of the HBoV-positive children. We found IgM antibodies and HBoV2 DNA in the sera of three children with AGE (one child having also respiratory symptoms), but in all cases norovirus was also detected in stools. Similarly, three patients with AGE as the main symptom and HBoV1-specific IgM and DNA in serum had norovirus, rotavirus or astrovirus detected in stools, as had also the one case with HBoV3 viremia. So even in the cases with acute HBoV infection, confirmed by IgM antibodies and HBoV DNA in serum, the symptoms of AGE were apparently caused by a virus other than HBoV. Whether HBoVs increased the severity of the disease in these double infections compared to infections without HBoV cannot be concluded.

In conclusion, while HBoV2 and HBoV3 were commonly found in stool samples, the findings could not be causally linked to AGE in hospitalized children. Whether these viruses actually multiply in the gastrointestinal tract could not be determined in this study. As for HBoV1, the association with respiratory tract infection was confirmed, but HBoV1 was detected in stools in addition to the respiratory tract, suggesting survival in passage through the gastrointestinal tract. The same should also be true for HBoV2 and HBoV3.

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The authors declare that they have no conflict of interest.

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Table 1. Human bocavirus (HBoV) DNA-positive findings in stool and nasal swab samples and combined (positive stool and/or nasal swab) results in the different study groups.

	Stool samples				Nasal swab samples				Combined results			
	AGE	ARTI	AGE/ARTI	*p-value*	AGE	ARTI	AGE/ARTI	*p-value*	AGE	ARTI	AGE/ARTI	*p-value*
HBoV1	2 **(1.2%)**	29 **(5.3%)**	18 **(7.6%)**	*0.014**	2 **(1.2%)**	23 **(4.2%)**	16 **(6.7%)**	*0.025**	3 **(1.7%)**	34 **(6.2%)**	22 **(9.2%)**	*0.008**
HBoV2	10 **(5.8%)**	28 **(5.1%)**	13 **(5.5%)**	*0.941**	1 **(0.6%)**	2 **(0.4%)**	0	*0.571***	10 **(5.8%)**	28 **(5.1%)**	13 **(5.5%)**	*0.941**
HBoV3	1 **(0.6%)**	6 **(1.1%)**	2 **(0.8%)**	*1.000***	0	0	0		1 **(0.6%)**	6 **(1.1%)**	2 **(0.8%)**	*1.000***
All	172	545	238		172	545	238		172	545	238	

AGE = acute gastroenteritis, ARTI = acute respiratory tract infection, AGE/ARTI = symptoms of both respiratory and gastrointestinal infection

*= Chi-Square test

**= Fisher´s exact test

Table 2. Details of the cases with acute human bocavirus (HBoV) infection, determined by finding of specific IgM antibodies.

					PCR findings			Other viruses in stool			
Study group	Gender	Age	Diagnosis*	IgM	*stool*	*swab*	*serum*	*rota*	*noro*	*astro*	*adeno***
AGE	female	16 mo	A08.4	**HBoV2**	**HBoV2**	neg	**HBoV2**	neg	**pos**	neg	neg
AGE	male	11 mo	A09	**HBoV2**	**HBoV2**	neg	**HBoV2**	neg	**pos**	neg	neg
ARTI	male	23 mo	J21.9	**HBoV1**	**HBoV2**	neg	neg	NA	NA	neg	neg
AGE/ARTI	male	19 mo	A09	**HBoV1**	**HBoV1**	neg	**HBoV1**	neg	neg	**pos**	neg
AGE/ARTI	male	20 mo	J22	**HBoV2**	**HBoV2**	neg	**HBoV2**	neg	**pos**	neg	neg
AGE/ARTI	male	18 mo	A08.4	**HBoV1**	neg	**HBoV1**	**HBoV1**	neg	**pos**	NA	NA
AGE/ARTI	male	23 mo	J21.9	**HBoV1**	**HBoV1**	**HBoV1**	**HBoV1**	neg	neg	neg	neg
AGE/ARTI	male	2 yrs 9 mo	A08.4	**HBoV1**	**HBoV1**	**HBoV1**	**HBoV1**	**pos**	neg	NA	NA
AGE/ARTI	female	3 yrs 4 mo	J18.9	**HBoV1**	**HBoV1**	**HBoV1**	**HBoV1**	neg	neg	neg	neg
AGE/ARTI	female	4 yrs	J18.9	**HBoV1**	**HBoV1**	neg	neg	neg	neg	neg	neg

*Diagnosis by ICD-10 codes (in the AGE/ARTI group this is the principal diagnosis/diagnosis of dominating symptoms):

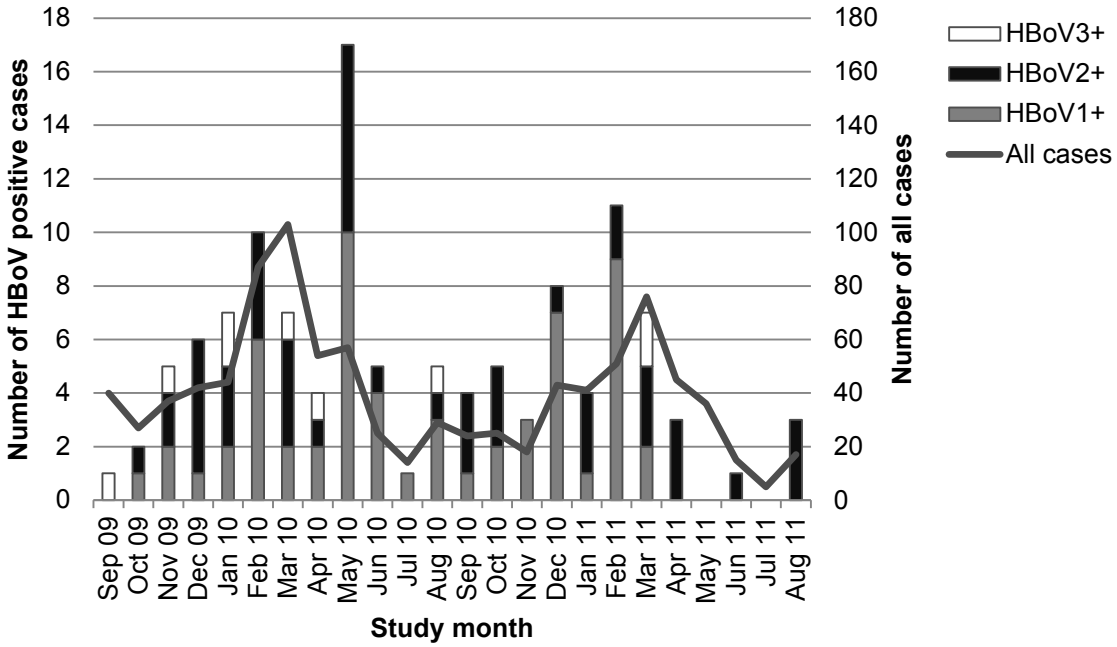
A08.4, unspecified viral intestinal infection; A09, other gastroenteritis and colitis of infectious and unspecified origin;

J22, unspecified acute lower respiratory infection;J21.9, unspecified acute bronchiolitis; J18.9, unspecified pneumonia

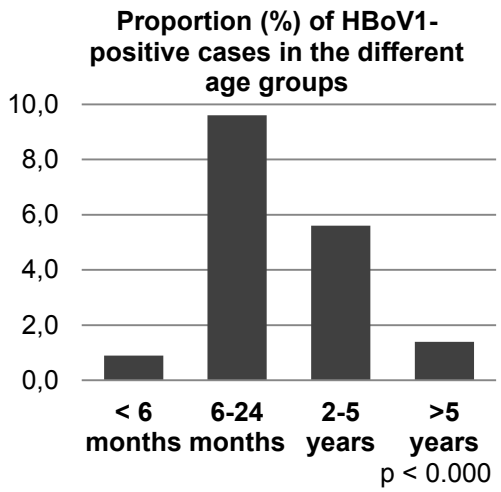
** Enteric adenovirus

419 **Fig. 1** The seasonal distribution of human bocavirus (HBoV)-positive cases and all cases studied.

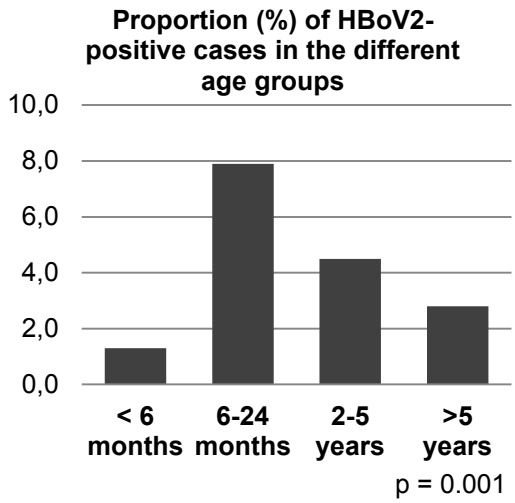
420 **Fig. 2** Proportions of human bocavirus (HBoV) types 1 and 2 (detected by PCR) in the different age
421 groups.


Number of HBoV positive cases
Number of all cases
Study month
HBoV3+
HBoV2+
HBoV1+
All cases
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
120
140
160
180
Sep 09
Oct 09
Nov 09
Dec 09
Jan 10
Feb 10
Mar 10
Apr 10
May 10
Jun 10
Jul 10
Aug 10
Sep 10
Oct 10
Nov 10
Dec 10
Jan 11
Feb 11
Mar 11
Apr 11
May 11
Jun 11
Jul 11
Aug 11

a)


Proportion (%) of HBoV1-positive cases in the different age groups
10,0
8,0
6,0
4,0
2,0
0,0
< 6 months
6-24 months
2-5 years
>5 years
p < 0.000

b)


Proportion (%) of HBoV2-positive cases in the different age groups
10,0
8,0
6,0
4,0
2,0
0,0
< 6 months
6-24 months
2-5 years
>5 years
p = 0.001