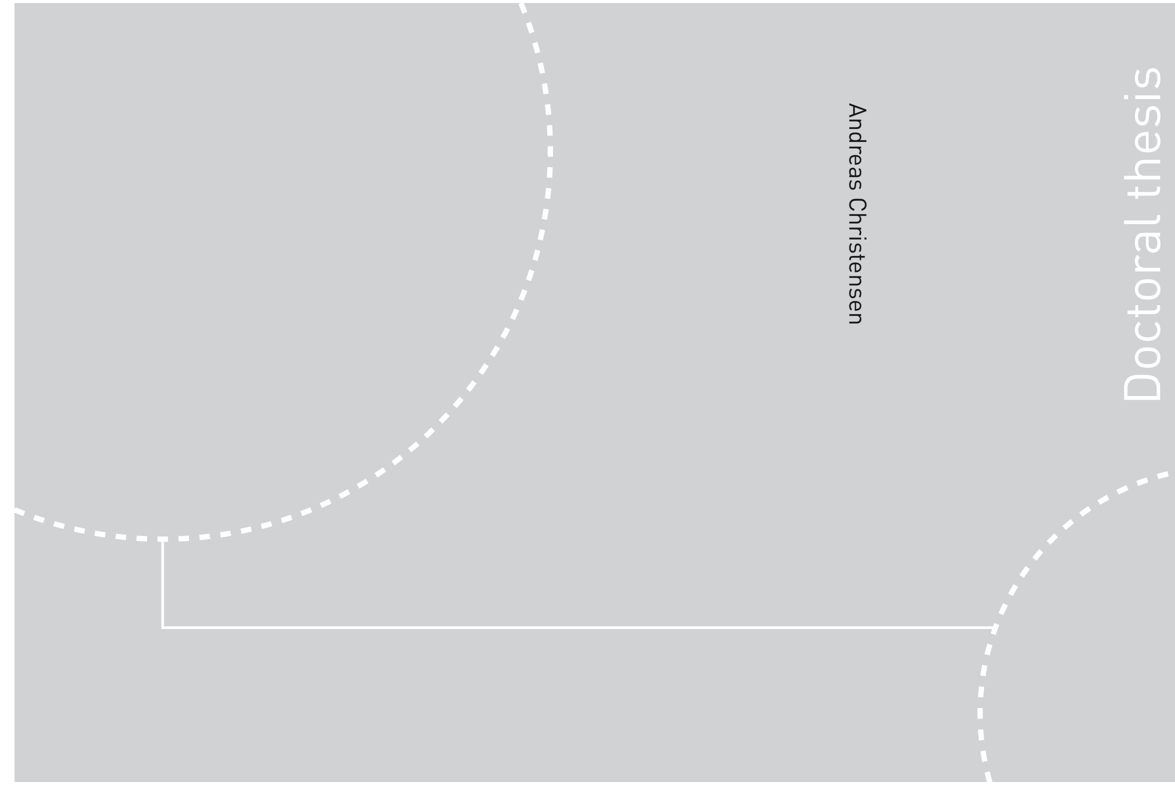

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Causal role of human bocavirus 1 in respiratory tract infections in children

NTNU
Norwegian University of Science and Technology
Thesis for the Degree of Philosophiae Doctor
Faculty of Medicine
Department of Laboratory Medicine, Children's and Women's Health

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Trondheim, June 2016

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Norsk sammendrag:

Humant bocavirus 1 og luftveisinfeksjoner hos barn

Humant bocavirus 1 (HBoV1) ble oppdaget i 2005 i luftveisprøver fra barn med luftveisinfeksjon. Det tilhører familien *Parvoviridae*, og var det første viruset i denne familien som man har assosiert med luftveisinfeksjoner hos mennesker. Senere studier har vist at viruset er vanlig også hos friske barn, og at det ofte påvises sammen med andre virus. Det har derfor vært tvil knyttet til om viruset spiller noen rolle ved luftveisinfeksjoner. Formålet med dette prosjektet har vært å se nærmere på om det kan være en årsakssammenheng mellom HBoV1-infeksjon og luftveisinfeksjoner hos barn. Vi har basert oss på materiale fra det pågående prosjektet «Childhood Airway Infection Research» (CAIR), og planla å studere assosiasjoner mellom ulike virusmarkører og luftveisinfeksjoner hos barn. Markørene var viruskonsentrasjon, påvisning av viruset alene (monodeteksjon), påvisning av viruset i blod (viremi) og viralt mRNA (markør for aktivt formerende virus).

Vi fant at HBoV1 var blant de hyppigst påviste virus hos barn yngre enn to år, og at det var det viruset som hyppigst ble funnet sammen med andre virus. Rundt 10% av neseprøvene fra barn innlagt med luftveisinfeksjon var positive for HBoV1, og i 75% av tilfellene ble minst ett virus påvist i tillegg (artikkel I og II). I andre artikkel viste vi at viruset var like hyppig hos barn med og uten luftveisinfeksjon, men subgruppeanalyser avslørte interessante sammenhenger. Påvisning av HBoV1 i blod var sterkt forbundet med luftveisinfeksjon. Høy virusmengde eller påvisning av viruset alene i neseprøve var også forbundet med slik infeksjon, men denne sammenhengen var svakere. I tredje artikkel viste vi at et nytt testprinsipp for påvisning av mRNA fungerte bra, med svært god analytisk sensitivitet og spesifisitet. I tillegg kunne vi vise at HBoV1-mRNA forekom i neseprøver hos 25 % av de HBoV1-positive barna med luftveisinfeksjon og hos ingen av kontrollene.

Resultatene støtter hypotesen om at HBoV1 forårsaker luftveisinfeksjoner hos barn. De utgjør en viktig del av et stadig økende evidensmateriale som støtter en slik årsakssammenheng. I tillegg kan vi konkludere med at tradisjonell, kvalitativ HBoV1-DNA-PCR er lite egnet i diagnostikken av HBoV1-infeksjoner hos barn.

Studien var basert på CAIR-prosjektet som startet i 2006 og fortsatt pågår. Som ledd i prosjektet ble prøver fra nesesvelget/nesen og, hvis mulig, blodprøver samlet inn fra alle barn innlagt med luftveisinfeksjoner ved Avdeling for barnesykdommer, St. Olavs Hospital,

Trondheim. I juni 2015 bestod materialet av 3285 neseprøver fra pasienter inkludert med samtykke fra pårørende. I tillegg var 628 kontrollprøver fra barn innlagt til elektiv kirurgi, uten symptomer på luftveisinfeksjon, inkludert. Artikkel I-III i avhandlingen er basert på et suksessivt økende grunnlagsmateriale etter hvert som CAIR-prosjektet økte i omfang. Henholdsvis 376, 1316 og 2379 neseprøver ble inkludert i studiene. Alle prøvene ble undersøkt med sanntids-polymerasekjedereaksjonstester (PCR'er) for 13-15 luftveisvirus og tre luftveispatogene bakterier. I tillegg ble prøvene dyrket for bakterier og virus. I siste artikkel beskrives et nytt testprinsipp for påvisning av virusspesifikt mRNA. Testen ble benyttet til å undersøke et materiale bestående av 161 neseprøver fra barn (133 pasienter og 28 kontroller) tidligere funnet positive for HBoV1 med tradisjonell HBoV1-PCR.

Navn kandidat: Andreas Christensen

Institutt: Institutt for laboratoriemedisin, barne- og kvinnesykdommer

Veileder(e): Henrik Døllner og Svein Arne Nordbø

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This work was supported by a grant from the collaborative organ between the Central Norway Regional Health Authority and The Faculty of Medicine of the Norwegian University of Science and Technology (NTNU). It was carried out in cooperation with the Department of Laboratory Medicine, Children's and Women's health at the Faculty of Medicine, NTNU and the Department of Medical Microbiology, St. Olav's University Hospital. I am grateful to these institutions for supporting my grant application and for all support during the research process.

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List of papers

Paper I

Human bocavirus commonly involved in multiple viral airway infections.

Christensen A, Nordbø SA, Krokstad S, Rognlien AGW, Døllner H.

J Clin Virol 2008; 41: 34-7.

Paper II

Human bocavirus in children: mono-detection, high viral load and viraemia are associated with respiratory tract infection.

Christensen, A., Nordbø SA, Krokstad S, Rognlien AGW, Døllner H.

J Clin Virol 2011; 49: 158-162.

Paper III

Detection of spliced mRNA from human bocavirus 1 in clinical samples from children with respiratory tract infections.

Christensen A, Døllner H, Skanke LH, Krokstad S, Moe N, Nordbø SA.

Emerg Infect Dis 2013; 19: 574-80.

Abbreviations

AOM	Acute Otitis Media
CAIR	Childhood Airway Infection Research Project
cDNA	Complementary DNA
CI	Confidence Interval
CPAP	Continuous Positive Airway Pressure
CPE	Cytopathogenic Effect
CRP	C-reactive Protein
CSF	Cerebrospinal Fluid
Ct-value	Cycle threshold value
ELISA	Enzyme Linked Immunosorbent Assay
GBoV	Gorilla bocavirus
HAdV	Human adenovirus
HBoV	Human bocavirus
HMPV	Human metapneumovirus
HRSV	Human respiratory syncytial virus
HRV	Human rhinovirus
LRTI	Lower Respiratory Tract Infection
MERS	Middle East Respiratory Syndrome
mRNA	Messenger-RNA
NP	Nucleoprotein
NPA	Nasopharyngeal Aspirate
NS	Non-Structural Protein
OR	Odds Ratio
RTI	Respiratory Tract Infection
RT-PCR	Reverse Transcriptase-PCR
SARS	Severe Acute Respiratory Syndrome
URTI	Upper Respiratory Tract Infection
VP	Viral Protein

Summary

Background

Human bocavirus 1 (HBoV1) was discovered in 2005 in respiratory samples from children with respiratory tract infections (RTI). It belongs to the *Parvoviridae* family, and was the first virus in this family to be associated with RTI in humans. Subsequent studies showed that the virus is common among healthy children, and that it is frequently detected together with other viruses. This has led many to question the virus' role in RTIs.

Aims

The aim of this project was to study the causal role of HBoV1 in RTI in children through the use of samples from the ongoing Childhood Airway Infection Research project (CAIR). We wanted to investigate associations between specific viral markers and RTI. These markers were viral load, detection of the virus alone (monodetection), viremia and viral mRNA.

Materials and methods

The CAIR project started in 2006 and is still ongoing. As part of the project, nasopharyngeal aspirates (NPAs) from all children admitted with RTI to the Pediatric department at St. Olavs Hospital in Trondheim, Norway were obtained, and if possible, blood samples were drawn. By June 2015, the material consisted of 3285 NPA samples from patients included with parental consent. Additionally, 628 control samples from children without respiratory tract symptoms admitted for elective surgery had been included. Papers I-III in this thesis are based on successively larger source materials as the CAIR project progressed; 376, 1316 and 2379 NPA samples, respectively. All samples were tested with real-time PCRs specific for 13-15 respiratory viruses and three respiratory bacteria. In addition, the samples were cultured for viruses and bacteria. First, a cross-sectional epidemiological study on 376 children admitted for RTI was carried out. Second, a study including a control group was conducted, encompassing 1154 children with RTI and 162 controls. The last study led to the development of a novel reverse transcriptase HBoV1 mRNA PCR based on primers spanning a splice site in the HBoV1 genome. The test was used to study 161 NPA samples from children (133 patients and 28 controls) which previously had tested positive with traditional HBoV1 DNA PCR.

Results

HBoV1 was among the most common viruses found in children under two years of age, and it was the virus most commonly detected together with other viruses. In our studies it was found in about 10% of the patients, and in 75% of the cases at least one other virus was detected (Papers I and II). The second study, which included a control group showed that the virus was equally common among children with and without RTI, but subgroup-analyses revealed interesting new patterns (Paper II). Adjusted for age and gender, HBoV1 detected alone in NPA was associated with RTI. Adjusted for age, gender and other viruses, a high HBoV1 load in NPA was associated with lower RTI (LRTI). Furthermore, viremia was found in 45% of children with RTI and in none of the controls. The third study showed that the detection principle based on a PCR spanning a splice site worked well, with excellent analytical sensitivity and specificity. Furthermore, detection of HBoV1 mRNA in NPA was strongly associated with RTI in children. It was found in 25% of children with RTI and in none of the controls.

Conclusions

We found significant associations between RTI in children and HBoV1 load, HBoV1 monodetection and presence of HBoV1 mRNA in NPA. Additionally, a strong association was found for HBoV1-viremia. The results support the hypothesis that HBoV1 causes RTI in children. The body of evidence which supports a causal link is substantially strengthened by our research. Furthermore, we can conclude that a qualitative HBoV1 DNA PCR is not suitable for diagnosing HBoV1 infections in children.

1 Introduction

Over the past two decades, we have witnessed a revolution in molecular genetics. The sequencing of the complete human genome, for instance, was finished in 2006. This was a landmark effort which had taken 16 years to complete. Sequencing technology has improved enormously since, and it is now possible to sequence a complete human genome within hours. This has led to burgeoning genetic research, and this development is still proceeding at a rapid rate. New genes and new organisms are constantly being discovered. The new technology has made it possible to detect microorganisms with much higher sensitivity and precision than before, and many organisms that were previously undetectable with traditional culture techniques have been discovered. Examples include bacteria such as *Tropheryma whipplei*, and viruses such as KI polyomavirus, WU polyomavirus, several picornaviruses and bocaviruses. Human bocavirus 1 (HBoV1) was discovered by the use of sequencing and cloning techniques in 2005. It was found in nasopharyngeal aspirates (NPAs) from children with respiratory tract infections (RTIs) (1). After discoveries like this it can be challenging to evaluate possible pathogenic roles of the new viruses. Large patient groups are needed for such research, which makes it time consuming and resource intensive. For HBoV1, additional challenges have appeared. The virus is often found together with other pathogenic viruses in children with RTI, and it is even common among healthy children. This has led many to suggest that the virus might just be an innocent bystander, not causing disease at all. In this thesis I will present three studies addressing these questions for HBoV1. The studies are based on the ongoing prospective project “Childhood Airway Infection Research” (CAIR) at our hospital. A general discussion on the causal link between the virus and RTI will follow. In the introduction, I will start by giving an overview of respiratory viruses and RTIs in general, followed by a review of current knowledge about HBoV1. I will place special emphasis on the detection of respiratory viruses in healthy children and on infections with multiple viruses.

1.1 Viral respiratory tract infections in children

1.1.1 Epidemiology

Respiratory tract infections are responsible for the highest disease burden of all diseases globally. The incidence rates are high in all parts of the world, but mortality rates are substantially higher in the developing world (2, 3). Incidence of severe pneumonia during the first five years of life has been estimated at 7.3 episodes per 1000 child per year in Europe, and 19.7 in developing countries (4). For children less than 5 years it is the leading cause of

death globally (5). Less severe diseases such as the common cold and bronchiolitis also represent a great burden on society, by causing discomfort in children and parental absence from work. Around 3% of all infants are hospitalized annually for bronchiolitis in western countries (6, 7), and during the first year of their lives children experience five to ten respiratory tract infections (8-10).

The majority of respiratory tract infections (RTI) in children are caused by viruses. This is especially pronounced for children under the age of five (11-16). Bacteria are frequently detected in respiratory samples, but their clinical significance is often difficult to interpret due to the bacteria's role in the normal flora of the upper respiratory tract. Clinically relevant detections of bacteria constitute only a fraction of the total number of bacterial detections in the upper respiratory tract (17). Although more rare than viral RTIs, taking the higher morbidity and mortality of bacterial pneumonias into account, bacterial RTIs' contribution to the total burden of disease is substantial (18).

1.1.2 Molecular epidemiology

Respiratory viruses are a very diverse and dynamic group of viruses. Large populations can be infected rapidly and viruses change constantly. This is most pronounced for RNA viruses such as, for example, the influenza virus. RNA genomes are generally less stable than DNA genomes and have a higher mutation frequency (19). Transmission via the respiratory route is effective through direct contact or via droplets propelled by coughing or sneezing. In a susceptible host population this type of virus can spread widely and cause an epidemic, which will subside when immunity in the population has reached a certain level. In a situation like this, selection for viruses with high mutation or recombination rates occurs. This helps them stay ahead of the hosts' immune responses. This can also explain why respiratory RNA viruses are so diverse, and why it has proven difficult to develop effective vaccines to beat them. Newly emerged viruses such as SARS-coronavirus, influenza A H5N1-virus and MERS-coronavirus all exemplify the versatile nature of RNA viruses. DNA viruses such as human adenovirus (HAdV) and HBoV1 are less prone to mutation, but may compensate for this by genome recombination (20). Another survival factor for respiratory DNA viruses might be an ability to exist dormantly/latently inside host cells, thereby escaping the immune response. This has been demonstrated for HAdVs (21, 22).

New diagnostic opportunities offered by modern gene technology have yielded possibilities to diagnose the majority of RTIs. Many studies have now revealed that children are exposed to a

large variety of viruses, and that many symptomless children do actually carry viruses (9, 23-25). In addition, many children harbor multiple viruses at the same time (26-28). HBoV1, rhinoviruses (HRVs), enteroviruses and HAdVs dominate as co-detected viruses, and HBoV1 is most commonly detected together with other viruses in respiratory samples. This raises diagnostic challenges: Does the virus cause disease, or is it just a bystander to other infections? As I mentioned above, I will address these questions later in this thesis, with a focus on HBoV1. Before I continue my review of RTIs in general, I will give a brief overview of the spectrum of viruses and bacteria which are associated with RTIs in children.

1.2 Virus families associated with respiratory tract infections

Respiratory tract infections are caused by a highly diverse group of viruses, including both RNA- and DNA viruses. I will briefly describe the most important families below.

1.2.1 Orthomyxoviridae

The family *Orthomyxoviridae* contains the human pathogen influenza virus which is divided into three genera: *Influenzavirus A, B* and *C*. The first two are well known causes of annual epidemics of influenza like illness in all age groups while species within *Influenzavirus C* cause milder RTI, mainly in children (29). Influenza A viruses can infect a variety of birds and mammals while influenza B and C viruses are solely human pathogens.

Influenza viruses are medium-sized viruses with a diameter of 100-120 nm. They are enveloped (surrounded by a lipid membrane) and their genomes consist of single stranded negative sense RNA. In addition, their genomes are segmented (7-8 segments). This special feature increases the viruses' ability to exchange genes (recombine). Occasionally, this can result in a so-called antigenic shifts leading to major global outbreaks, so called pandemics. Humans have low immunity against these antigenically new viruses leading to increased morbidity, increased viral shedding and consequently increased viral transmission. This has happened five times during the last century (30).

Regular seasonal influenza is usually milder than pandemic influenza, but does cause considerable morbidity and mortality (31, 32) Common symptoms are fever, joint pain, muscle tenderness, a runny nose and coughing. Viral pneumonia is common in the more severe cases, and this predisposes for bacterial pneumonia (33). Severe cases are most common among children, pregnant women, the elderly and people with chronic diseases. Viral RNA can be detected in the nasopharynx from one day before until one week after the

acute illness. This coincides with the period when the patient is contagious (34). Immunity against the same virus strain lasts for a few years. However, because the viruses change constantly, protective immunity may be low even after one year.

Compared to other respiratory viruses, influenza viruses are quite uncommon among children. Influenza A and B viruses are detected in 1-4% of children with RTI (9, 26, 27, 35).

1.2.2 Paramyxoviridae

Paramyxoviridae is a large family within the order *mononegavirales*. It contains well-known human pathogens such as the parainfluenza virus, human respiratory syncytial virus (HRSV), measles virus, mumps virus and the more recently discovered human metapneumovirus (HMPV). Today, morbillivirus and mumps virus are rare in the Nordic countries because of effective vaccine programs, but the other viruses are all common respiratory pathogens, especially in children.

The *Paramyxoviridae* are large enveloped viruses with a diameter of 150-350 nm. Their genomes consist of single-stranded negative sense RNAs similar to influenza virus, but the RNA molecules are not segmented. As RNA viruses, they are constantly mutating, undergoing antigenic changes. However, having single RNA molecules, they are not able to recombine to such an extent as influenza viruses. The surface molecules on paramyxoviruses are, as a consequence, more stable. This means that our immunity against these viruses can last longer. Adults are consequently less prone to infections from these viruses than children. HRSV infection is the classic example predominantly infecting very young children, and seldom adults (26, 36).

HRSV appears in annual epidemics among small children. They typically start in winter and last for three to four months. The pattern is less repetitive for HMPV and parainfluenza viruses. HMPV can occur in epidemics but less frequently than HRSV, often every second to third year (37). Parainfluenza viruses appear annually in smaller outbreaks during autumn or winter, or sporadically. HRSV is usually among the most common viruses detected in children with RTI, with detection rates from 10 to 30% (9, 27, 36). HMPV and parainfluenza viruses are rarer with occurrences around 2 to 6% (9, 27, 38). Prevalence depends on season, and to reduce this effect I have only referred to studies performed over longer periods covering at least two years.

Clinical manifestations associated with paramyxovirus infections are highly variable, ranging from the common cold to serious lower respiratory tract infections (LRTI) needing intensive care. The typical syndrome associated with HRSV is bronchiolitis with respiratory obstruction. HMPV causes less respiratory obstruction than HRSV, but on the other hand, it is more strongly associated with interstitial pneumonia (39, 40). Parainfluenza viruses usually cause milder symptoms, and are frequently associated with laryngitis and hoarseness (41). Gene material from paramyxoviruses is usually detectable in nasopharyngeal aspirates from one day before and one to two weeks after the acute illness. (42, 43)

1.2.3 Picornaviridae

Picornaviridae constitute another large and diverse virus family. It contains the well-known enteroviruses (which include the polioviruses), HRVs and the recently named parechoviruses. Today, the polioviruses are eradicated from Europe and will not be discussed further in this thesis. Their presence in other parts of the world, however, makes them still relevant in a global perspective. Currently, there are a few hundred subtypes of both HRVs and enteroviruses, and they are constantly evolving (44). As for most respiratory viruses, picornaviruses primarily infect young children, but adults are afflicted to a greater extent than for paramyxoviruses (45). Both HRVs and enteroviruses appear in annual waves with peaks in the period from September to December (9, 46, 47). In addition, they are sporadically detected all year round, and for HRVs, a second wave in spring may occur. In children with RTI, HRVs are generally the most common viruses detected, with rates between 15 and 48% (9, 27, 45). Enteroviruses are also common (3-20%) but their role in RTI has not been resolved (26, 48, 49).

Picornaviruses are non-enveloped small viruses with a diameter of 28-30 nm containing a single-stranded linear positive sense RNA genome. They are robust viruses and the enteroviruses in particular are able to stay infective for days outside a human host (50, 51).

HRVs are the classic «common cold-viruses», but there is increasing evidence for a role of these viruses in LRTI in both children and adults (28, 52, 53). In addition, HRVs are the viruses most strongly associated with asthma in older children (54). I will discuss the link between HRVs and asthma in section 1.7. Enteroviruses may cause RTI, but other diseases are more common (46). These include viral meningitis, sepsis-like manifestations in newborns and illnesses dominated by fever and rashes in children and young adults. In addition, symptomless infections are common for both enteroviruses and HRVs (25, 55).

Parechoviruses and enteroviruses are closely related, and they cause a similar spectrum of diseases (56). However, most parechoviruses were discovered recently and their clinical relevance remains to be established.

Diagnosis of picornavirus infections is complicated by the common presence of asymptomatic infections. In addition, long time shedding of enteroviruses has been documented (48). For HRVs, the duration of shedding is shorter, generally less than two weeks (52, 57, 58). Overall, it is challenging to separate the clinically relevant virus detections from silent presence of viral genomes. We have similar challenges with HBoV, and I will discuss this issue more thoroughly in the main part of this thesis.

1.2.4 Coronaviridae

Four species within the family *Coronaviridae* are well-known causes of respiratory tract infections in humans. These are named human coronavirus OC42, human coronavirus 229E, human coronavirus NL63 and human coronavirus HKU1. The nomenclature is based on labels given by different independent research groups, and for this reason it is somewhat confusing.

Coronavirus infections can appear in outbreaks, but mainly occur sporadically in autumn, winter or spring (59, 60). In hospitalised children, coronaviruses usually constitute a modest fraction of the viral detections with rates in the range 2-7% (61, 62). Many coronaviruses have been detected in mammals, especially in bats, which may be a common source of infection for other animals including humans (63). Bats were the probable source in 2002/2003, when the SARS-coronavirus suddenly appeared in humans causing a rapid and deadly epidemic (64). The virus has, however, not been detected since. In 2012 a similar event took place in Saudi Arabia with the appearance of the MERS-coronavirus. This time, the immediate source may have been dromedary camels. The outbreak is still going on but has not reached the proportions the SARS epidemic had. To date, it has mainly been confined to the Arabian Peninsula, and the disease is less dramatic in otherwise healthy patients (65, 66).

Cornaviruses are medium-sized enveloped viruses with a diameter of 120-160 nm. They have a single-stranded linear unsegmented positive sense RNA-genome. With a size of 32 kilobases, it is the largest known viral RNA-genome.

Coronaviruses have mainly been associated with milder RTI (60, 67, 68), but SARS and MERS-coronavirus have shown us that viruses within the family may cause serious life-

threatening respiratory tract infections. Coronavirus NL63 has in addition been associated with laryngitis in children (69).

RNA from coronaviruses can be detected in nasal secretion from day 0 until day 10 after symptom start, and for SARS- and probably MERS-coronavirus it is characteristic that infectivity is low before symptomatic infection is established (70).

1.2.5 Adenoviridae

HAdVs have since the 1960's been well established as respiratory pathogens. They are divided into six species named A to F, and further subdivided into more than 70 types. The different types may cause a wide range of clinical conditions, from RTI, to keratitis and gastroenteritis. Types 1-7 are the ones most often associated with RTI.

The respiratory HAdVs occur worldwide and sporadically all year round. Children less than five years of age are those mainly affected, but adults and especially patients with immunosuppressive conditions may also be infected.

HAdVs are among the few respiratory viruses that has a DNA genome. The virus particles are icosahedral and non-enveloped with a diameter of 80 nm. They have medium-sized genomes for a DNA virus, about 35 kilobase-pairs long. The genome is double-stranded, linear and codes for approximately 40 proteins.

HAdVs are mainly associated with mild upper RTI, but severe pneumonias occur. Certain genotypes (type 7 and 14) have been associated with severe disease (71, 72). In addition, severe HAdV infections are a well-known complication in post-transplant patients (73).

DNA from HAdVs can be detected in nasopharyngeal samples for weeks after resolution of symptomatic infection (74). It is also frequently detected in asymptomatic children without apparent recent disease (25). Prolonged replication of the virus may explain both features. Reactivation of latent infection is an alternative explanation when the virus is detected in asymptomatic children, or in children infected with other respiratory viruses. Such reactivation has been demonstrated in lymphocytes isolated from human adenoid tissue (21). This creates diagnostic problems similar to those described for HRVs and HBoV1. It is difficult to tell whether an HAdV detection is related to the disease in question, or whether it is a coincidental finding during a disease of different causes. A live vaccine against HAdV types 4 and 7 has been developed, but it is not available in Norway.

1.2.6 Parvoviridae

In this virus family, only HBoV1 has been associated with RTI in children. The discovery of this virus was published in 2005 (1). Viruses in this family are small non-enveloped viruses with a diameter of 25 nm. They contain single-stranded DNA coding for three or four proteins.

HBoV1 was detected in nasopharyngeal samples from children with RTI, and evidence has accumulated since, supporting a causal link between this virus and RTI in children. I will come back to this in detail later.

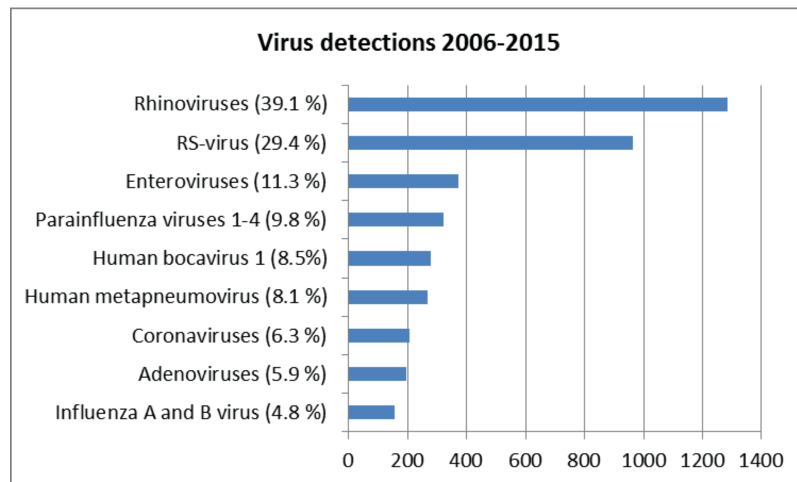

Figure 1. Viruses detected in NPAs from children 0-15 years old in the CAIR project 2006-2015, distributed by numbers of detections. Percentages are shown in parentheses.

1.3 Role of bacteria in respiratory tract infections in children

The focus of this thesis is community acquired viral infections in the nose, throat, bronchi and lungs of children. In this section I will briefly discuss bacteria's role in this context. Streptococcus *pneumoniae* (pneumococci), *Haemophilus influenzae* and *Moraxella catharralis* are the bacteria most often associated with community acquired RTI in children, and pneumococci are the most commonly detected bacteria in respiratory samples (16, 75, 76). *Staphylococcus aureus* and *Streptococcus pyogenes* are rarer causes of community

acquired RTI in children (14, 77, 78). Bacteria usually cause pneumonia dominated by fever, fatigue and varying degrees of coughing. Characteristically, temperature, serum C-reactive protein (CRP) and leucocyte numbers are high, higher than with viral RTI, and the disease is usually more severe (18). The clinical distinction between bacterial and viral RTIs can nevertheless be difficult. In addition, the bacteria mentioned above can all be part of the normal human bacterial flora. Pneumococci, for instance, can be detected in more than half of healthy children, and similar figures are found for *H. influenza* and *M. catharralis* (79-81). This makes clinical evaluation of bacterial detections in nasopharyngeal samples difficult. Other diagnostic procedures such as blood culture or bronchoscopy are required for finding the etiological agents. The diagnostic performances of these methods are, however, also low, due either to low sensitivity (blood culture) or to high frequency of contamination from upper airway flora (bronchioalveolar lavage) (82-84). For these reasons, the etiological agents of bacterial RTIs are usually not found, making it hard to obtain good epidemiological data about bacterial RTIs. Better occurrence estimates can be gained by the use of aspirates obtained through needles inserted directly through the chest wall. A few such studies have been performed, revealing that pneumococci are present in ~30% of the cases and *Haemophilus influenzae* in ~10% of the cases (85, 86). Very low occurrence rates have been registered for other bacteria, but in studies including tests for *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*, these bacteria were detected in approximately 20% and 10% of the cases, respectively (87). It is important to keep in mind that the studies were primarily performed on adults and that the study populations were highly selected, consisting of patients with severe pneumonias and localized infiltrates on chest radiograms. More reliable occurrence data can also be obtained by studying host immune responses against the bacteria. A Finnish study from 2003, documented that antibodies against pneumococci were present in 28 % of children with pneumonia (75). However, this study was performed on material from 1981-1982, long before the introduction of a pneumococcus-vaccine in the national childhood vaccination program in Finland. This vaccine was introduced in Norway in 2006, and the occurrence of invasive pneumococcal infections and pneumonias has declined since then (88, 89).

The bacteria *Bordetella pertussis*, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* are not considered part of the normal human flora, and the evaluation of positive test results on nasal or pharyngeal samples is easier. *Bordetella pertussis* causes whooping cough, which can be very distinct clinically with intense paroxysms of coughing. However, milder variants

dominated by runny nose, pharyngitis and only a slight fever, similar to a viral infection, occur. Children routinely receive pertussis vaccines in most of the western world, and the milder variant is especially common among slightly older vaccinated children (90). *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* cause very similar diseases. Symptoms are dominated by a nonproductive dry cough often lasting for weeks. The disease is commonly called “atypical pneumonia”. “Atypical” here means less severe pneumonia but often of longer duration. It is most common among adults but can also occur in small children (91).

Bacteria cause RTIs by direct invasion of the airways. We have very little knowledge about why this happens in only a subset of infected or colonized people. Co-infecting viruses may play a role. Interactions between viruses and bacteria have been widely discussed since the 1918 influenza pandemic when numerous cases of secondary bacterial pneumonia were observed. We know today that influenza infection increases the likelihood of subsequent pneumococcal pneumonia, and we have good evidence that supports a causal link (33). Interactions between viruses and bacteria may be subtle and species specific, but more general mechanisms are probably also involved (92). These include epithelial dysfunction leading to mucus stagnation and epithelial destruction increasing the bacteria’s access to respiratory tissue.

1.4 Seasonality of viral respiratory tract infections

Most respiratory viruses show distinct seasonal fluctuations in temperate climate zones (9, 16, 35, 93). Figure 2 shows the seasonal pattern for some viruses included in the CAIR project. The clearest example of a seasonal virus is HRSV which appears in distinct annual winter, or early spring, epidemics in small children. Influenza appears in equally regular epidemics. The other viruses all show seasonal patterns of varying degrees. A special pattern can be seen for HRVs which usually give two peaks annually, one in early autumn and the next in winter. The first peak has been linked to sudden crowding of children indoors at school start in August/September (94). The DNA viruses HBoV1 and HAdV appear to have the least pronounced variations with only slight increases in winter.

The reasons for the seasonal fluctuations are still debated. Correlations between low temperature, low relative humidity and increases in RTI have been documented a number of times (95), but the question of whether these features actually cause increased RTI frequency is difficult to answer. In a Finnish study from 2009, RTI was significantly associated with

lower temperatures and lower relative humidity during the three days preceding infection. Moreover, an association was also found for significant decreases in both parameters during the three days (96). This temporal pattern hints at a causal link. A few earlier experimental animal studies support these findings (97, 98), and a recent study indicates that reduced innate immune responses at lower temperatures play a causal role (99). Peaks in RTI occurrence are also associated with indoor crowding, but the question of causality is also here unresolved (100).

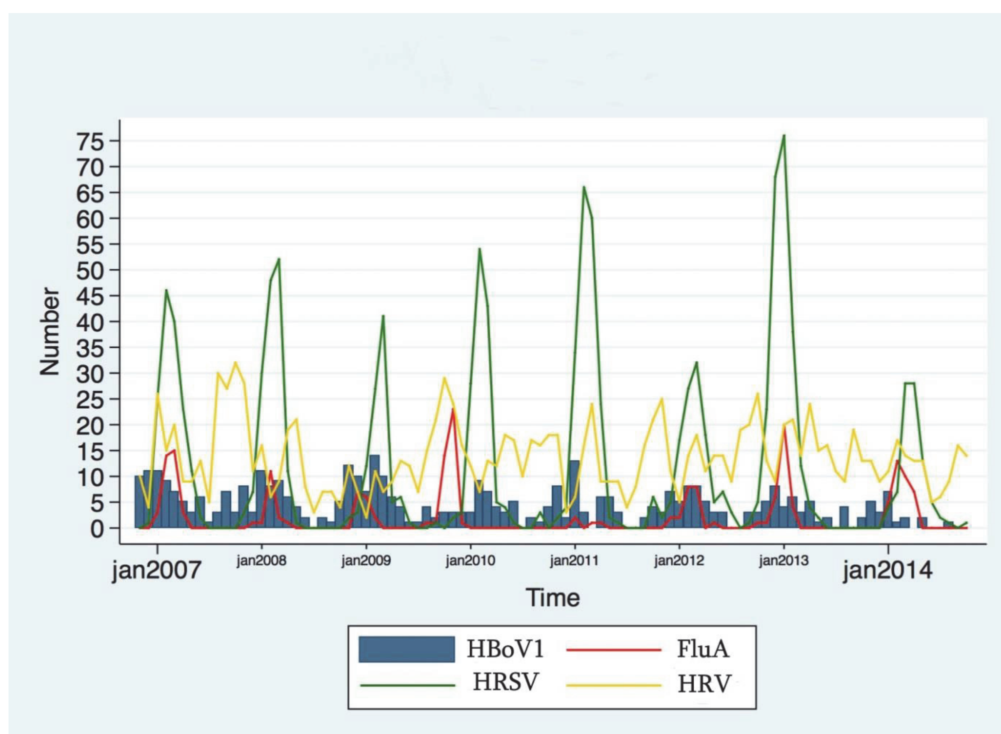

Figure 2. Monthly distribution of HBoV1 detections in NPAs from children in the CAIR project 2007-2014. Distributions of HRSV, Influenza A virus (FluA) and HRV are included for comparison.

1.5 Transmission

Although the viruses causing RTI differ considerably genetically and structurally, their transmission patterns are very similar. Most studies indicate that droplet transmission is their main route of transmission (45, 101-104). This means that direct contact with a patient or the secretions from a patient is necessary for transmission to occur. "Droplet transmission" means virus transmission via droplets propelled through air by for instance a sneeze and not via dry airborne particles. The measles virus, which is a paramyxovirus, is an exception. It can spread

by airborne transmission. There is, however, some data indicating that influenza viruses spread more effectively than the other respiratory viruses and that airborne transmission may play a role for them (105).

Most RTI patients are infectious for a day or two before symptoms appear and again a few days onwards. Children generally shed viruses for a longer time than adults. Shedding of influenza virus in children has been demonstrated from two days before the onset of symptoms and until seven days after (34). A Finnish study on HRV transmission within families showed that children could shed the virus for up to two weeks. Furthermore, they found that transmission rates were very high for children under seven years of age, and that asymptomatic infections were common among adults. Transmission of infection from symptomless individuals infected with HRV was not demonstrated (23).

Transmission of respiratory viruses in hospitals is a recurring problem during the winter months, especially in pediatric wards. HRSV dominates causing up to half of RTIs in hospitalized children under five years of age at this time of the year (26, 106). In children with malignant or immunosuppressive diseases, HRSV can cause severe and even fatal infections. Early diagnosis and adequate infection control measures are therefore of critical importance.

1.6 Clinical features of respiratory tract infections in children

In this section, I will take a closer look at the different clinical syndromes associated with respiratory infections in children. I will discuss their definitions and highlight some diagnostic problems of which it is important to be aware when discussing the clinical relevance of HBoV1.

Respiratory infections are mainly classified according to their localization along the respiratory tract. The division into upper and lower respiratory tract infections (URTI and LRTI) is a rough but practical classification, giving information about the severity of disease. LRTIs are usually the more severe ones (107). In the papers included in this thesis, I have used this classification.

1.6.1 Upper respiratory tract infections

The common cold is an acute infection of the nasal and pharyngeal mucosa leading to rhinitis and pharyngitis, respectively. Symptoms are nasal congestion, runny nose and more variably

sore throat. Cough, hoarseness, fever, headache and malaise are also frequent symptoms. Upon examination, inflamed nasal and pharyngeal mucosae are found, sometimes accompanied by inflammation of the eye (conjunctivitis) or tympanic inflammation (simplex otitis). All viruses mentioned in the previous sections may cause this syndrome, but HRVs are clearly the most common (108, 109).

Acute tonsillitis, or pharyngotonsillitis, is often, and especially in adults, a distinct clinical syndrome with few of the typical symptoms seen with the common cold. The disease is characterized by fever, sore throat and swollen tonsils leading to dysphagia. Streptococcus pyogenes is the main bacterial cause, in which case tonsillar exudates are common. Frequent viral causes are the Epstein-Barr virus (a virus belonging to the herpesvirus group), HAdVs and enteroviruses. Parainfluenza virus, HRV, coronavirus and HRSV may also cause the syndrome, but then usually less pronounced and in addition accompanied by symptoms seen with the common cold.

"Laryngitis" or "laryngotracheitis" are overlapping terms referring to infection and inflammation in and around the larynx. The term "croup" is used when inflammation in the larynx area is accompanied by inspiratory stridor. In small children, it is characterized by fever, a typically a barking cough and, per definition, inspiratory stridor. In older children, the characteristic cough is less prevalent, but hoarseness is more common. Laryngitis may lead to serious respiratory obstruction and the need for prompt treatment (110-112).

Upper respiratory tract infections are usually self-limiting and pass within a week. Prolonged disease may be seen with serious underlying diseases or with suppurative complications. A common complication in small children is bacterial infection of the middle ear (otitis media) leading to earache and a red bulging tympanic membrane upon inspection. In older children and adults, sinusitis is a more common complication, characterized by facial pain or headache and varying degrees of fatigue.

1.6.2 Lower respiratory tract infections

When an infection reaches the lower respiratory tract, severe cough is characteristic, often accompanied by fever and pronounced fatigue (10). Depending on whether the bronchi, the bronchioles and the lung parenchyma are involved, the terms "bronchitis", "bronchiolitis" and "pneumonia" are used.
The terms "bronchitis" and "bronchiolitis" refer to infection and inflammation of the larger

bronchi or the smaller bronchioles respectively. Children less than two years of age have narrower bronchioles with less rigid walls than older children. This leads to a stronger tendency to respiratory obstruction during LRTIs. The association with age is so strong that ages lower than two years have been included as a diagnostic criterion in many countries, including Norway. Bronchiolitis is defined as respiratory tract infection with dyspnea and/or a chest X-ray showing hyperinflation or peribronchial thickening in children under two years of age (113). Symptoms from the upper respiratory tract are usually present but this is not a diagnostic criterion. The disease can be indistinguishable from an asthma attack. I will discuss the distinction between bronchiolitis and asthma later.

In older children, the term "bronchitis" is used, and this syndrome is mainly characterized by severe coughing and fever in addition to varying symptoms from the upper respiratory tract. Airway obstruction leading to dyspnea is less common in this age group, but when it is present, the term "obstructive bronchitis" is used.

Bronchioloitis is a serious childhood disease, common in all parts of the world. It is a leading cause of hospitalization in the western world with estimated admission rates between 24 and 55 per 1000 children under 1 year (114, 115). Median duration of symptoms for bronchiolitis is approximately two weeks, indicating that the burden of disease is considerable (116). In one study, median reported days missed at daycare was 2,5 days per disease episode (117). In a Canadian study 16% of children hospitalized for bronchiolitis were in need of intensive care support (118), but mortality is low in the western world, estimated to 2 per 100 000 child years (119).

RSV is the most frequent causative agent of bronchiolitis, but many other viruses may cause similar symptoms. These include parainfluenza virus, HAdV, HRV, HMPV and coronaviruses (39, 120).

Pneumonia is defined as infection and inflammation of the lung parenchyma. It is often divided into community- and hospital-acquired pneumonia. Hospital-acquired pneumonia is defined as pneumonia arising after 48 hours in hospital. This distinction is important because the two entities are caused by different spectra of microbial agents leading to different treatment strategies. The subject of this thesis is community acquired respiratory infections, and I will focus on this in the following. Another important distinction is whether the pneumonia is caused by bacteria or viruses. In contrast to bronchitis or bronchiolitis, bacteria are common causes of pneumonia. Bacterial pneumonias need prompt treatment with

antibiotics; hence, it is important to diagnose these infections early, but the distinction can be difficult to assess. First, good microbiological samples are difficult to obtain from sites deep in the lungs. Second, the diagnostic accuracy of radiological examinations is low. Classic bacterial consolidations on radiographs may not be produced by all bacterial agents, and may sometimes be produced by viruses (121, 122). Manual and auscultatory examination of the patients may give helpful additional information, but it is largely subjective and seldom conclusive (123). A high fever >39°C, high levels of CRP and high numbers of leucocytes in blood are indicative of bacterial pneumonia (124, 125), but many other diseases may give similar findings. Consequently, in clinical practice, the diagnosis of pneumonia is made discretionary, based on an overall evaluation of the information above. In medical research, stricter criteria are usually needed. In our study, viral pneumonia was defined by symptoms and signs suggestive of pneumonia (fever, fatigue, tachypnea, retractions, nasal flaring, crackles, decreased breath sounds), consolidations or signs of pleural effusion on chest X-ray and serum CRP <70 mg/L and/or 3-12 x 10^9 leucocytes/L blood. The definition of bacterial pneumonia was based on the same clinical and radiological criteria, but with a serum CRP > 70 mg/L and/or < 3 or > 12 x 10^9 leucocytes/L blood. This approach should give the best combination of sensitivity and specificity for both viral and bacterial pneumonia (123, 126). In the retrospective part of the study, three trained nurses and four MDs, all involved in the project, made the diagnostic evaluations.

Pneumonia definitions differ considerably from study to study. In resource-limited situations, for example, it is important not to oversee patients with serious infections. Very inclusive criteria are consequently used. This gives a high sensitivity but a poorer specificity, leading to a high number of unnecessary treatments. The WHO criteria are an example, with a calculated low specificity (127). In the western world, advanced diagnostic procedures are available, and a stronger focus on specificity can be justified. When evaluating the pneumonia literature, it is important to consider these differences.

Pneumonia is a serious disease common in all parts of the world, and is, as mentioned, the leading cause of death in children globally (5). Bacterial pneumonia is the main contributor to high mortality, but viral pneumonia may also have serious consequences. Many studies indicate that HRSV and HMPV are associated with the most severe diseases, with higher hospitalization rates, longer hospital stays and higher fever (9, 128-130). Certain types of HAdV (types 7 and 14) are also associated with severe pneumonia (71, 72). Of host factors, prematurity and low age (0-5 months) are important risk factors for serious LRTIs with

HMPV and HRSV (131). Immunsuppressive underlying diseases such as leukemias have also been shown to increase mortality considerably with certain respiratory viruses. The best documentation exists for HRSV and parainfluenzaviruses (132-134).

1.7 Viral respiratory tract infections and asthma in children

Viral bronchiolitis causes symptoms very similar to an acute asthma attack, and it is a long-debated question in medicine whether respiratory tract infections in childhood cause subsequent development of asthma. Asthma is defined as repeated episodes of airway obstruction. At least three episodes in less than a year is a requirement for the diagnosis, but as low as one episode is accepted when the child shows symptoms or signs of allergy or atopy. There are numerous triggers for asthma attacks, and viral infections are among them. This makes studies on causality difficult. Are infections the primary cause leading to lasting changes in the respiratory tract, or are the viruses just bystanders to a disease process of other causes? A few studies published in the last two decades have addressed these questions, and we are starting to see the outlines of some answers. It is well established that HRSV- and HRV infections during the first three years of life are significantly associated with asthma development a few years later (54, 135). The association is strongest at age seven, and according to one study recedes towards the teens (136). However, in a Swedish long term follow-up study, the association remained until age 18 (137). These studies are cohort studies showing associations, but they do not answer questions on causality. The affected children might have underlying conditions predisposing them to both infections and asthma development.

Intervention studies are needed in order to address the causality question. If prevention of infections could reduce the occurrence of asthma, it would support the hypothesis that there is a causal link between early infections and the later development of asthma. We currently have no effective means of preventing HRV infections, but there is a monoclonal antibody preparation, palivizumab, available that does reduce HRSV morbidity (138). A few studies using palivizumab in this context have been published, and they do show an effect on later asthma occurrence. A recent randomized study showed a relative reduction of 61% in number of days with wheezing during the first year of life (139). The follow-up time was very short, and we have to await further results from the study group. In an earlier study, a similarly preventive effect of palivizumab until four years after cessation of treatment was

demonstrated. However, this study was only performed on premature children, which is a subgroup of children with a very special risk profile, and it was not randomized (140). Furthermore, the study showed that the effect was only demonstrable for children with neither a family history of atopy nor atopy themselves. These studies support the idea that HRSV infections are involved in the pathogenesis of asthma, but the dependence on atopy hints at a more complex picture where both host factors and viruses play roles. In a recent study, this was explored further (141). Caliskan et al identified certain polymorphisms in the human gene locus 17q21 as risk factors for asthma after HRV infection. A number of experimental animal studies supporting this view have been published (142-146). It seems that the interferon response is an important determinant of long-term airway responsiveness. In rat models, an impaired interferon gamma response leads to long-term hyper-responsiveness in the respiratory tract after acute viral infections. Infants have weaker interferon responses than older children and adults, and this may partly explain why viral infections, especially in early childhood, are so strongly associated with asthma development.

In conclusion, both viral and host factors seem to be important for the development of asthma. A combination of genetic predispositions and exposure to viruses, especially HRV and HRSV, determine the progress to chronic disease. Prevention of viral respiratory tract infections may reduce the occurrence of childhood asthma. In the future, it will be of interest to study possible associations between asthma and other respiratory viruses, including HBoV1.

1.8 Occurrence of respiratory viruses among healthy children

After the introduction of sensitive molecular detection methods such as the polymerase chain reaction (PCR), detection rates for respiratory viruses have increased considerably. Very high rates, up to 68%, have even been found among asymptomatic children (25, 27, 147-149). In most studies, HRVs and HBoV1 stand out as the dominant ones among healthy children, and this has raised concerns about causality for these viruses. Despite this, there are good arguments supporting causal roles for the viruses. I will discuss the causality question for HBoV1 later.

What do the respiratory viruses found in healthy children represent? False positive results due to technical issues, such as contamination, are one possibility. Modern laboratories have

extensive routines for avoiding this, but the possibility must always be considered. Having ruled this out, I will list four biological explanations. The viral nucleic acids may represent: 1) remnants of a previous infection, possibly undergone weeks ago, 2) symptomless infection, either acute or reactivated, 3) acute symptomatic infection but the symptoms are not recognized or reported or 4) infection under development, not yet symptomatic.

Long term shedding of viruses is a requirement for the first hypothesis. Shedding for weeks and even months has been demonstrated for HBoV1 and enteroviruses, and is a plausible explanation for these viruses (150-152). Detection of nucleic acids from coronaviruses has also been demonstrated three weeks after the acute infection, for a subset of the infections (93). Most other viruses, however, are shed for shorter periods (days) (28), and the probability is therefore lower for detecting these viruses in aymptomatic individuals. Positive HRV PCR results have also been documented for weeks after an acute infection, but two Finnish studies have shown that this is probably caused by asymptomatic reinfections with new virus strains (23, 52). Consequently, the second explanation (symptomless acute infection) is plausible for this virus group. Asymptomatic reactivation of latent viruses has been shown for HAdVs (21, 153), and is under discussion for HBoV1 (152). The third hypothesis applies to all virus groups. Mild symptoms may not be recognized by parents or caregivers. It is hard to control for this in a study setting, and one has to keep in mind that a certain percentage of controls may actually be mildly symptomatic. Hypothesis 4 also applies to all the viruses. An asymptomatic patient might be in the incubation period and develop symptoms after the consultation. In one study, four out of 29 (14%) asymptomatic children with viruses detected in NPA developed symptoms within two weeks (52). The same viruses were found at follow up.

For HRVs, coronaviruses, parainfluenzaviruses and HBoV1 it has been demonstrated that viral loads are lower in asymptomatic than symptomatic children (27, 148, 154) (paper II). This supports the hypothesis that asymptomatic individuals positive for these viruses have undergone an infection, mild or asymptomatic, and that the viral genomes detected are remnants of a previous infection (hypotheses 1 or 2). Studies on viral dynamics during infections with HRSV and influenza virus on volunteers show a sharp increase in viral load the day before symptoms appear and a slower decline in the days thereafter, indicating that the most probable explanation is a previous infection rather than an infection in the incubation period (155). For HRVs, however, conflicting results on viral load in asymptomatic children

have been reported (23, 156). This is a complex and diverse group of viruses, and further studies based on thorough subtyping are warranted.

According to our CAIR data, the most common viruses found in asymptomatic children are, HRVs, enteroviruses, HAdVs and HBoV1. In contrast, the viruses HRSV, HMPV and influenza viruses are rare among asymptomatic children (unpublished data).

1.9 Multiple viral infections

In addition to uncovering viruses in healthy children, new molecular methods have made the identification of multiple different viruses in large proportions of NPAs from children possible. The term "co-infection" has often been used in this context to specify the presence of a virus in addition to other viruses. This may be an inaccurate term because we do not know whether the detected virus is part of a true infection, or whether viral nucleic acids are present only as, for example, remnants of a previous infection. The term "co-detection" has been introduced as a more inclusive term, not dividing between active replication and the mere presence of viral nucleic acids. I find it appropriate to use this term, unless it is clear that I am discussing productive infections involving replicating viruses.

In epidemiological studies based on molecular detection methods, at least two viruses have been found in 17-40% of the children with RTI (15, 26-28, 93, 108, 148, 157, 158), and in a few patients, as many as six different viruses have been detected. In studies including a control group, about a third of the healthy children were virus-positive, and 12-22% of these contained multiple viruses (27, 148). Most studies have been performed on children less than two years of age. Cilla et al found a significantly higher proportion of multiple infections among children <12 months than >12 months (15). These data illustrate the heavy infectious pressure on the youngest children. Many viruses circulate all year round among these children, giving both asymptomatic and symptomatic infections. The viruses most frequently involved in multiple infections are the ones most frequently detected in general. This is as expected, but HBoV1 stands out as particularly common. More than two thirds of all HBoV1 detections have been made in patients whose NPAs contain additional viruses (26, 159) (paper I). I will discuss this phenomenon later. It is our experience that HAdVs also stand out in this context (unpublished data). The prevalence of HAdV has been highly variable in studies performed but in a few other studies with high HAdV occurrence, an association with co-detections was observed, meaning that the frequency of HAdV detections was higher among children with other viral infections than in the total material (108, 157, 160).

Detection of multiple respiratory viruses in a patient with RTI makes clinical evaluation difficult. Are all viruses involved in the disease process or are some of them “innocent” bystanders? Frequent detection of respiratory viruses in healthy children suggests that some co-detected viruses are clinically irrelevant. In addition, the frequent detection of multiple viruses raises questions about possible synergistic or antagonistic interactions between them. From a clinical and diagnostic point of view, it is natural to ask the following questions: First, are certain combinations of viruses more common than others? Second, do some of the combinations lead to more or less severe disease than when the viruses occur alone?

A co-detected virus may represent a productive, symptomatic infection, added to another infection. It may contribute to the symptoms, but this will depend on the symptoms normally associated with the viruses involved, and whether interactions occur. On the other hand, co-detected viruses may also represent innocent bystanders, meaning they are not involved in the pathogenic processes. Explanations similar to the ones suggested for asymptomatic infections in the previous section may be valid in this context also. The co-detected viruses may be part of asymptomatic productive infections (primary or reactivated), remnants of previous infections or they may represent infections in the incubation period. In addition, viral genomes present in cells may be released during a new infection with a different virus which causes cell lysis. Another explanation worth considering is whether or not some co-detected viruses primarily act as co-factors or risk factors for disease, meaning that they are not sufficient causes of disease but may contribute to it, when they interact with other infectious agents.

Studies on interactions between viruses, and between viruses and bacteria are difficult to perform - mainly because of the multitude of possible combinations of agents. Between 20 and 30 respiratory agents have been identified so far. In addition, some of them, such as HRVs and enteroviruses, are subdivided into up to a few hundred different subtypes. With such diversity, it is difficult to design accurate tests - tests that include only the target viruses and exclude other related viruses. In this situation, inter-laboratory variations in test design may influence the results and cause biases. The detail level of the analyses may also influence results. In some studies, the term “co-infection” has merely been used without specifying the co-detected viruses. (28, 161-163). This creates opportunities for bias. Others have performed detailed analyses on specific interactions between pairs of viruses. It is necessary to do the analyses at this level to take into account each virus’ specific properties. For example, if influenza A-virus is co-detected in HRSV-infected patients a higher rate of fever will be

expected. Such studies are demanding. Extensive materials are required to give adequate statistical power to studies on infrequent combinations. Unsurprisingly, interaction studies performed thus far have given conflicting results. HRSV is the most common virus in many hospital-based materials, and most studies have consequently involved this virus. In one study, detection of both HRSV and HMPV was associated with higher bronchiolitis severity than when each of the viruses was detected alone (164). However, in two other studies, this association was not found (165, 166). Furthermore, two studies focusing on HRSV and HRVs found no interactions between the viruses in terms of disease severity (167, 168). In another study, researchers compared children infected with HRSV alone with children infected with HRSV in combination with other viruses. They found that the latter group had a higher frequency of fever and stayed longer in hospital than the former (169). They used logistic regression analyses in order to control for some biases, but important ones remained. Influenza and HMPV were common among the co-detected viruses. This gives a plausible explanation for the difference. Both viruses are associated with high fever in children. A few studies addressing specific interactions between HBoV1 and other viruses have been performed. They will be discussed below in section 1.13.2.4.

Interactions between viruses and bacteria have also been investigated in a few studies. Detection of both influenza A-virus and Staphylococcus aureus in respiratory secretions from children has been associated with higher rates of admissions to intensive care units, and even higher fatality rates than for both agents alone (170). As mentioned in section 1.3, an association between influenza A infection and pneumococcal pneumonia has been documented. The virus predisposes for a bacterial superinfection. A similar but weaker effect was shown for parainfluenza viruses. These are accentuating effects of the viruses on the pneumococci. Effects in the opposite direction, that pneumococcal infections may accentuate influenza or parainfluenza virus infections, have not been documented. Nevertheless, there are important links between these infectious agents.

At present, viral load seems to be the best parameter for evaluating clinical relevance of co-detected viruses. As mentioned above, viral load for HAdVs, HRVs, coronaviruses, parainfluenza viruses and HBoV1 is usually lower in asymptomatic than symptomatic patients. If these viruses are detected in multiply infected individuals, a low viral load will indicate low clinical relevance, i.e. they are probably "bystanders". According to data from the CAIR material, viral load is less important for HRSV, HMPV and influenza virus, and the presence of these viruses must be considered clinically relevant, regardless of viral load

(unpublished data). The infection dynamics are different for each of the respiratory viruses and need to be carefully studied in order to establish practical cut-off values that can help clinicians in making decisions about clinical relevance.

1.10 Microbiological diagnosis

Microbiological laboratories have traditionally played a minor role in the diagnosis of respiratory tract infections. This is partly due to the good prognosis of most of these infections, and that criteria for diagnosing serious cases in need of anti-bacterial treatment have principally been based on clinical information and biochemical tests results. In addition, low test performance has been a problem. Both sensitivity and specificity have been low for most of the relevant traditional microbiological tests. However, with the introduction of modern molecular methods, this has changed dramatically. I will now briefly go through the methods in use, both classical and modern, before I give a short discussion about the current role of microbiological diagnostics for respiratory tract infections in children.

1.10.1 Viral culture

Methods to sustain and propagate live cells in nutrition-rich media were developed in the 1960s. In this way, researchers could culture both human and animal cells in the laboratory. The breakthrough in virological research came when methods to cultivate cells in monolayers on glass or plastic surfaces were introduced. This made it easier to observe pathogenic effects on cells and cell clusters – so called cytopathogenic effects (CPE). Based on CPE-pattern, identification of viruses to group level became possible with regular light microscopy. By the additional use of virus specific antibodies, viruses could be identified to both species and sub-type levels. The method was very specific for viruses that grow well in cell cultures, but sensitivity varied. A wide spectrum of viruses did not grow in cultures at all. In addition, growth was often slow and would take from a few days up to a couple of weeks before CPE was visible. Low sensitivity and slow speed both led to the abandonment of the method in clinical laboratories when modern molecular methods were introduced. However, viral culture is still a cornerstone in modern virological research, and it is my opinion that larger clinical laboratories should keep the method as backup and a corrective to modern molecular methods, as molecular methods need constant follow up and regular updates. In addition, the method may facilitate new discoveries and unexpected findings. HMPV was, for example, discovered by coincidence in a monkey kidney cell line (171).

1.10.2 Antigen detection

A more rapid approach, detection of viral proteins without culturing, was developed in the 1970s. Viral proteins (antigens) were detected by immunological methods. Antibodies collected from animals challenged with viruses or viral proteins were used for detection of proteins in patient samples. The most widely used method today is enzyme linked immunosorbent assay (ELISA), although the method which represented the breakthrough was immunofluorescence. With these methods, specific binding between the viral protein and added antibodies was detected through easily visible color changes or fluorescence signals, respectively. Viruses in throat or nasopharyngeal samples could thereby be detected within hours, not days. This was a significant step at the time, but it has now become clear that the tests are suboptimal for most respiratory tract infections. The tests for HRSV perform satisfactory, but all other viruses (influenza virus, parainfluenza virus, enterovirus and HAdV) show low scores for sensitivity and specificity (172). Antigen detection was the basis for many studies on respiratory tract infections until the mid-2000s. It is important to have the limitations of the antigen tests in mind when evaluating these studies. Because of this diagnostic bias, they cannot be compared directly with studies based entirely on PCRs.

1.10.3 Antibody detection

Detection of antibodies is performed with the same immunological methods mentioned above, and ELISA is today the most common test principle. Predefined antigens were added to samples and the presence of specific antibodies detected by changes in color. The most commonly used material is serum. This method became widely used in the 1980s, and was very useful for a number of infectious diseases, especially HIV and hepatitis viruses, and remains so today. However, the quality was unfortunately low for respiratory tract infections (172). Serological tests for influenza viruses, parainfluenza viruses, HRSV, HAdVs, *Mycoplasma pneumonia*, *Chlamydophila pneumonia* and *Bordetella pertussis* were all hampered by low sensitivity and specificity. An additional problem with antibody tests is slow conversion to positivity. The tests become positive one to four weeks (sometimes later) after symptom debut. This means that the infections are often diagnosed after they have resolved. Despite the broad availability of PCR tests in the western world, antibody detection is still in use by many physicians and laboratories, although their use is declining.

1.10.4 Nucleic acid detection

New molecular detection methods and primarily the polymerase chain reaction (PCR) have revolutionized medical microbiology since its introduction in the late 1980s. The method is based on sequence specific amplification of small stretches of gene material (RNA/DNA) from viruses, bacteria or parasites. The method enables highly sensitive and specific detection of the infectious agents, and it has made possible the discovery of previously unknown pathogens. HBoV1 is one such example. PCR has expanded the field of respiratory tract infections considerably. Only a small fraction of respiratory tract infections were known before the PCR era. In earlier studies, maximally 40% of respiratory tract infections in children were diagnosed, but after the introduction of PCR, it has become possible to diagnose more than 90% (172). With PCR, we can identify infectious agents with high degrees of accuracy, but new problems have arisen. As already mentioned, many agents are detected together with other viruses or bacteria, and in addition, viruses are often detected in healthy individuals. This has raised questions about the clinical relevance of the detected viruses and bacteria. Detection of gene material from an agent does not necessarily mean that the agent is alive or causing infection. Research on these issues for respiratory viruses is today only in its infancy. Sequencing performed directly on samples, so-called deep sequencing or next generation sequencing, is another approach which gains more and more support as it becomes steadily more affordable. The role of such powerful methods in clinical virological diagnosis remains to be determined, but has great potential as an unbiased method facilitating unexpected detections.

1.10.5 Microbiological diagnosis of RTI in children today

Nasopharyngeal samples such as swabs or aspirates are preferred sample materials for PCR when viral RTI is suspected. Traditionally, viruses have not been expected to be present in healthy individuals, and detections of viruses in samples from the upper airways were thought to represent the causative agents, as is also the case for infections in the lower airways. Indeed, good correlations between detections in the nasopharynx and the lower airways have been demonstrated for most viruses and for *Mycoplasma pneumonia* (173). At least, viruses present in the lower respiratory tract are also usually present in the upper respiratory tract. On the other hand, HRVs, coronaviruses and human bocavirus are often only detected in the upper airways, and the detections are made in both symptomatic and healthy children (173, 174). Consequently, detections of these viruses in samples from the upper airways must be

evaluated with caution, especially in children with LRTI. For most bacterial LRTIs it is necessary to get material from the lower airways, preferably by bronchoscopy. If this is not feasible, expectorates may be used, and in that case, it is important to keep in mind that the material is often contaminated with flora from the upper airways. As described above in section 1.3, most of the relevant bacteria are part of the normal human pharyngeal flora, and their presence in this location may well be without relevance for the clinical condition.

PCR is a very powerful diagnostic tool, but does it give useful information to clinicians in their daily practice? Does a positive or a negative result influence clinical decisions? Early diagnosis gives the clinician an opportunity to start specific treatment early, but specific drugs are available for only a fraction of the infections: serious influenza and bacterial infections. In most cases, this means that the test results have few direct clinical consequences. It is usually enough to know whether or not an infection is bacterial. It has been common to use less costly biochemical tests such as CRP and clinical criteria rather than microbiological test in order to make this distinction. It is nevertheless important to keep in mind that diagnostic tests may also give important prognostic information. In cases where serious differential diagnoses such as cancers are under consideration, specific microbiological diagnoses may be of importance, even if antimicrobial treatment options are lacking. Knowing whether a viral infection is the probable cause of, for instance, bronchial inflammation rather than a malignant process will influence both treatment and investigation plans for the individual patient. There is still much we do not know about the clinical spectrum and normal course of the different viral respiratory tract infections, especially in patients with serious underlying diseases. With modern molecular methods, however, we now have the opportunity to study this in detail. This may lead to new indications for diagnosing viral respiratory tract infections in the future. Moreover, early diagnosis of viral infections has been assumed to reduce unnecessary treatments with antibacterial drugs. This would put patients at lower risk of side effects, and reduce the selection pressure for development of antibiotic resistance among bacteria. Many studies support this hypothesis (175-182), but a few recent studies have challenged this view (183-185). Cost-benefit analyses are needed before good practice recommendations can be given. On the cost side of the equation, we have the price of PCR tests. The price is high today, but going down rapidly. Furthermore, new antiviral drugs are constantly under development. The therapeutic outlook in this field may thus change in the near future, altering the benefit side of the equation.

1.11 Treatment and prevention

1.11.1 Prophylaxis

Prophylaxis through immunization is available against infections caused by *Bordetella pertussis*, pneumococci and influenza. In addition, vaccines against mumps, measles and diphtheria are available. These agents may also cause respiratory tract infections, but are rare today in Norway due to effective vaccination programs.

In order to reduce the infectious pressure in hospitals, contact precautions for droplet transmission are employed for children admitted with RTI. Hand washing between patient contacts is crucial. Washing with soap kills, for example, HRSV effectively (186). In addition, it is common to recommend the use of gloves and gowns when caring for infected patients, although the evidence for this is weaker. Co-isolation of infected children, so-called cohort isolation, is recommended when possible, but the scientific evidence supporting this is also weak. Moreover, HRSV specific antibodies can be administered as prophylaxis to vulnerable children during the HRSV season. This has been shown to reduce hospitalizations for HRSV in this patient group by 39-78% (138).

1.11.2 Treatment

For bacterial respiratory tract infections, the main rule is to treat with antibiotics as early as possible. The choice of drugs is dependent on the bacterium involved, emphasizing the importance of early microbiological diagnosis when bacterial infections are suspected. For serious influenza infections, there are two treatment options: oseltamivir and zanamivir. For all other viruses, no specific antiviral drugs with proven therapeutic effects are available, and physicians have to rely on supportive measures for seriously ill children. The aim of the treatment is to reduce respiratory distress by the use of bronchodilators, oxygen inhalation and, if necessary, supported respiration with continuous positive airway pressure (CPAP) or respirator (2).

1.12 Causality

I will end the general introduction with introducing some concepts on causality in relation to infectious diseases in general.

Robert Koch is generally credited for creating the most famous criteria used for establishing the etiology of a specific infectious disease. Based on his experiences with anthrax and

especially tuberculosis, he formulated a set of principles which have later been reformulated and termed Koch's postulates (187). One example is shown below.

Koch's postulates:

- The parasite (infectious agent) occurs in every case of the disease in question, and under circumstances which can account for the pathological changes and clinical course of the disease.
- The parasite occurs in no other disease as a fortuitous and non-pathogenic parasite.
- After being fully isolated from the body and repeatedly grown in pure culture, the parasite can induce the disease anew.

Many have also added that the agent should be isolated again from the body where the disease was induced anew.

Koch described a necessary and sufficient relation between cause and effect. This means there should be a one-to-one relationship between an infectious agent and the disease it produces. The association between for example, *Bacillus antracis* and anthrax is very close to this. Such relations are very distinct, relatively easy to study, and they were naturally the first to be discovered. It has later become clear that most infectious agents are involved in much more complex relationships with both other microbes and the host. Koch's postulates were effective as tools for convincing sceptics of the contagion hypothesis at the time, and they have been prudent guidelines for enthusiastic microbiologists, eager to discover new pathogenic microbes during the following century. However, it is generally agreed upon today that the postulates are too strict. We now know that many bacteria are part of the normal flora in healthy individuals and are only associated with disease in special cases. The disease may be caused by a change in the balance between the bacterium and the host's defense mechanisms, or by a change in the balance between the bacterium and other microbes. A network-based, ecological approach to the causality problem is now gaining support. On the one hand, a specific disease may have many causes, both infectious and non-infectious, on the other, a specific microbe may be involved in the pathogenesis of many diseases, but it may equally well not cause disease at all. This is far from the one-to-one relationship described by Koch. It is important to keep in mind that we are usually studying causal factors only, not the sole cause of a disease.

Different logical, practical and molecular approaches have been suggested to improve Koch's postulates, but it has proven difficult to provide criteria which are both specific, and which cover all cases (187-189). Published in 1965, the Bradford Hill criteria are among the most inclusive (190). They were defined as minimal criteria for establishing the etiology of any disease, not just infectious diseases. They are listed in table 1. They serve as good reminders of the multifactorial approach necessary in research on causality in medicine. For both technological and ethical reasons, it will often be impossible to give definitive proof for a causal relation between an infectious agent and a disease, and we will have to settle with building up a convincing body of evidence from many angles. In any case, the first step will be to establish whether there is a statistical association between an infectious agent and a disease. It is very important to only use the word "association", until the total body of evidence convinces the specialist community and finally scientists in general.

Table 1. A short version of the Bradford Hill criteria

Strength	A correlation with significant strength measured in odds ratio or relative risk must be present
Consistency	Reproducibility
Specificity	The disease should be less frequently seen when the proposed causal factor is absent
Temporality	The causal factor must precede the disease in time
Biological gradient	There should be a dose-response relationship between the causal factor and the disease
Plausibility	The causal explanation must make biological sense through a plausible mechanism
Coherence	The causal explanation should fit with current biological knowledge
Experiment	Manipulation experiments should change the outcome
Analogy	When an equivalent system is available the effects should be analogous in the two systems

In medical microbiology, the first goal is usually to find a reproducible statistical association between detection of a microbe and a disease. Detection of live microbes is optimal, but many newly discovered viruses and bacteria are only detectable with molecular methods. A dose-response effect, meaning that high loads of an organism are associated with more severe

disease, will strengthen the hypothesis for a causal link. Serological evidence showing an association between a specific antibody response against the agent and the disease will give additional support. This is an important argument to which I will return in the general discussion. Demonstration of the organism in the organ producing the most pronounced symptoms is also of value. Animal or cell models showing equivalent responses to those observed in humans are very important, if available. Such models will also allow for genetic manipulations in order to see if the loss of specific genes leads to loss of pathogenicity (loss of function-studies). This is often regarded as strong evidence for causality. Finally, intervention strategies through specific treatment or prevention will usually be appreciated as final evidence, if such studies show reduced morbidity or mortality. For some of the newly discovered viruses only detected with molecular methods, including HBoV1, many of these approaches are impossible. Both cell and animal models are, for example, lacking for HBoV1, and treatment options are not available. When we don't have experimental models available, the Bradford Hill criteria will be the most useful guidelines for discussing causality. They will be the basis for my final discussion.

1.13 HBoV1

The discovery of HBoV1 was published in 2005 by Tobias Allander's research group at Karolinska Institutet in Stockholm (1). They used a random amplification technique for viral genomes on nasopharyngeal aspirates from patients with respiratory tract infections. The amplified genomes were further characterized by sequencing after a cloning step. Along with many genomes from well-known viruses, an unknown parvovirus was found. Genetic analysis showed that it was closely related to the animal viruses, bovine parvovirus and minute virus of canines. The genus comprising these two viruses had the name bocavirus, and the new virus was named human bocavirus (HBoV), accordingly. Parvovirus taxonomy was revised in 2014 (191). An overview of current taxonomy is given in table 2. As the table shows, a number of bocaviruses have been detected in mammals as diverse as gorillas, pigs, sea lions, cats and dogs. Most of them have been detected during the last ten years. Moreover, studies on bocavirus-like-viruses found in bats, rats, red foxes and chimpanzees are under way. In addition to HBoV1, three other human bocaviruses have been found, named HBoV2-4. These are mainly found in human feces, and might be associated with gastroenteritis (192-194).

1.13.1 Taxonomy and basic virology

1.13.1.1 Taxonomy

HBoV1 belongs to the genus *Bocaparvovirus* in the subfamily *Parvovirinae* within the family *Parvoviridae*. Gorilla bocavirus (GBoV) is its closest relative found so far. HBoV1 and GBoV are grouped together within the same species, *Primate Bocaparvovirus 1,* alongside HBoV3. These three viruses are now to be regarded as different subspecies rather than species. GoBV was first detected in feces from gorillas with gastroenteritis, but the pathogenic potential of the virus has not been clarified (195).

Table 2. Current classification of viruses within the genus *Bocaparvovirus*

Genus	Species	Virus
Bocaparvovirus	*Carnivore bocaparvovirus 1*	canine minute virus
	Carnivore bocaparvovirus 2	canine bocavirus 1
	Carnivore bocaparvovirus 3	feline bocavirus
	Pinniped bocaparvovirus 1	California sea lion bocavirus 1
		California sea lion bocavirus 2
	Pinniped bocaparvovirus 2	California sea lion bocavirus 3
	Primate bocaparvovirus 1	human bocavirus 1
		human bocavirus 3
		gorilla bocavirus
	Primate bocaparvovirus 2	human bocavirus 2
		human bocavirus 4
	Ungulate bocaparvovirus 1	bovine parvovirus
	Ungulate bocaparvovirus 2	porcine bocavirus 1
		porcine bocavirus 2
		porcine bocavirus 6
	Ungulate bocaparvovirus 3	porcine bocavirus 5
	Ungulate bocaparvovirus 4	porcine bocavirus 7
	Ungulate bocaparvovirus 5	porcine bocavirus 3
		porcine bocavirus 4

1.13.1.2 Structure

As a typical parvovirus, HBoV1 is a small icosahedral non-enveloped virus with a diameter of about 25 nm. Its capsid consists of sixty units, each made from two structural proteins called viral protein 1 and 2 (VP1 and VP2). Studies on related parvoviruses, and on recombinant

HBoV1-like particles, indicate that VP2 is the main component of the capsid surface, making it the most exposed and immunogenic protein (196). The host's immune response is predominantly directed towards VP2 (197).

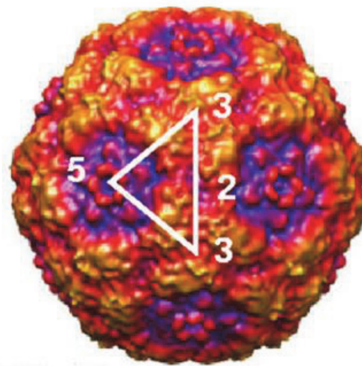

Figure 3. Model of an HBoV1 particle based on x-ray-crystallography studies. Colors indicate diameter, going from blue through red to yellow. The deepest canyons are marked blue and the highest peaks are marked yellow. Localizations of two, three and five-fold axes on the icosahedral structure are indicated. Reprinted from reference (196) with permission from the publisher.

1.13.1.3 Genome

The HBoV1 genome consists of single stranded, negative sense DNA with hairpin ends, which is well known from other parvoviruses (198, 199). It is approximately 5 300 bases long, and contains three open reading frames (ORFs), coding for four annotated (to date) proteins: NS1, NP1, VP1 and VP2 (Fig 4). As indicated above, the two latter proteins are structural proteins making up the viral capsid. In one study, a third VP3 protein was found, probably split off from VP2, but this has not yet been confirmed by others (200). NS1 and NP1 are non-structural proteins with multiple functions. NS1 (non-structural protein 1) is involved in viral DNA replication, and manipulation of the host cell cycle (201, 202). NP1 (nuclear phosphoprotein 1) is unique for bocaviruses and has been shown to be an important part of the viral replication machinery for the related minute virus of canines (198). For HBoV1, NP1 has been shown to facilitate nuclear localization of viral DNA, and it is probably also involved in modulation of the host immune response (203-205). In a recent study, three new variants of the NS1-gene produced by alternative splicing were identified and named NS2-4. Their functions are still unclear, but NS2 appears to be involved in viral replication (201). Cell models have been established for HBoV1 (201, 206-209). They are

based on primary human bronchial cells cultured in a liquid gas interface in order to mimic conditions in human airways. These cultures are labor intensive and often give little yield, but they have made gene expression studies on HBoV1 possible (206). Others have used recombinant viruses containing the HBoV1 genes in regular cell models with success (210). In figure 4, an overview of HBoV1 mRNAs taken from the latter paper is given. Later studies have, as mentioned, added detail to NS1-expression (201). It is clear that alternative splicing (alternative recombining of the mRNAs) is an important mechanism by which the virus increases the diversity of its proteins, as it is for all parvoviruses. In this way, it is possible to make at least ten different proteins from a genome only five kilobases long. This is a highly efficient use of genetic information, twice that of most bacteria. I will come back to the splicing phenomenon in paper III of this thesis.

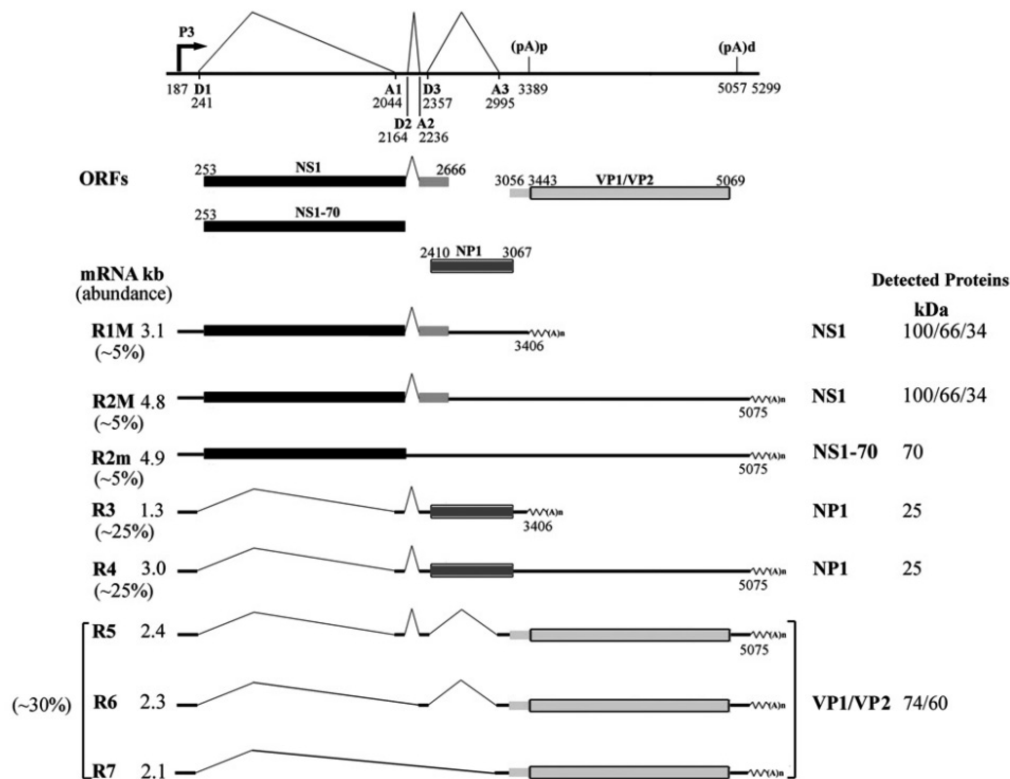

Figure 4. Summarized genetic map of HBoV1 with transcription profile. Size (kb) and relative abundance (%) of mRNAs are shown to the left. Protein designations and sizes (kDa) are shown to the right. Reprinted from reference (210) with permission from the publisher.

1.13.1.4 Cell tropism and infection dynamics

HBoV1 infects human respiratory tract cells in primary cell cultures and causes cytopathogenic effects compatible with acute respiratory infections (207). As mentioned, LRTI is common in children hospitalized with acute HBoV1 infection. This indicates that the virus is able to infect respiratory cells down to the bronchioles. The virus has also been detected in tonsillar tissue, but it is unclear which cell type they primarily infect there (24). Aside from this, we have little knowledge about HBoV1 cell tropism and entry. Virus is detectable in blood for one to two weeks after a primary infection and sometimes longer (211). Whether the virus infects other cells during this phase is unknown. HBoV1 DNA has been detected in urine and feces, but little is known about the role of these materials in viral transmission. Nasal or respiratory secretions are probably most important for transmission. A patient can shed viral DNA in respiratory secretions for weeks and even months after an acute infection (150, 152). This has raised the question of whether HBoV1 can establish persistent or latent infections. In 2011 two papers were published which added some support to this idea (212, 213). Both study groups had uncovered circular forms of the HBoV genome, Lusebrink et al in human respiratory cells (HBoV1) and Kapoor et al in ileal biopsies (HBoV3). These might be replicative intermediates (involved in viral reproduction) or so-called episomes. We know episomes from herpes viruses where they are important for latency. A recent Russian study has confirmed the findings for all human bocaviruses (214). These are highly interesting findings showing unique properties of human bocaviruses, but the biological function of the circular forms is still unclear.

The data we have so far on innate immune responses against HBoV1 infections are scarce. Expectedly, interferon and interleukin responses typical for viral infections are elicited, and one study has also shown upregulation of profibrotic cytokines (215, 216). Comparison with parvovirus B19 and HRSV has revealed some significant differences, but it is too early to draw any conclusions about the meaning of the differences, or whether there are unique features in the cytokine responses against HBoV1 (215, 217). In one study HBoV1 was shown not to induce programed cell death in respiratory cells (apoptosis) (210). This stands in contrast to parvovirus B19. Inducement of apoptosis is a well-known pathogenesis factor for this related virus. However, inducement of apoptosis in cervical cancer cells (HeLa cells) has been documented for the HBoV1 NP1 protein (205). It is uncertain what this finding in strongly aberrant cancer cells may mean for pathogenesis in the virus' normal target cells.

1.13.2 Epidemiology

After the discovery of HBoV1, a number of cross-sectional studies on HBoV1 occurrence were published. Different designs and sampling methods were used, but nevertheless a clear picture appeared: The virus is common in respiratory samples from children (218). It occurs globally, and is often among the three to four most common viruses detected in children with respiratory tract infections, with detection rates ranging from 2% to about 20% in hospital-based studies (159, 166, 219-232). Similar occurrences have been documented in the community (93, 148, 152, 233).

HBoV1 DNA is most commonly detected in small children. In the early hospital-based studies on children, median age was usually in the range of one to two years (219, 224, 228, 234, 235). In these studies, calculations of median ages were based on qualitative detection of HBoV1 DNA. We know today that long term shedding of HBoV1 DNA is common after an acute infection. This leads to a bias towards higher median ages. On the other hand, by using a material consisting of hospitalized children you obtain a bias towards the most serious infections. The smallest children are often the most severely affected, thereby creating a bias towards younger ages. In total, the effect becomes unpredictable. The median age for primary acute HBoV1 infections is best calculated through follow-up studies of children from birth until a few years of age. In a recent community based study performed by Martin et al, such a strategy was used, and the median age for the first detection of HBoV1 was 11 months (152). This is probably a more accurate estimate of median age for HBoV1 infections, and it illustrates that HBoV1 infection is a disease of early childhood. The youngest child infected was only two days old. They also found that 92% of all children studied had been infected by two years of age.

The HBoV1 prevalence in adults with RTI is considerably lower, with rates at around or below 1% (159, 224, 228, 231, 236). However, in a recent American study a somewhat higher prevalence was found (7% of samples from patients 18 to 39 years old) (233). Few studies on the elderly have been performed, but according to one extensive Chinese study HBoV1 seems to be equally uncommon among elderly as in adults (231).

Most HBoV1 studies have been hospital-based. As mentioned, one should be cautious about drawing conclusions about the epidemiology, including seasonal patterns, in such materials. HBov1 is frequently a co-detected virus, and during outbreaks with other viruses, the detection rate for HBoV1 will naturally increase if all samples are examined for this virus too.

Indeed, most studies have documented increased occurrences of HBoV1 during the winter season when infections with other viruses such as HRVs and HRSV increase (224, 225), but when controlled for number of samples taken, no significant seasonal variations are found (demonstrated in paper II of this thesis). A better picture of seasonality can be obtained through thorough follow up-studies. The aforementioned study by Martin et al did not find significant seasonal variations in acute primary HBoV1 infections. HBoV1 infections seem to occur sporadically all year round.

1.13.2.1 Serological studies

Serological studies have confirmed that HBoV1 is a common virus with high seroprevalence rates demonstrated in children from two years of age and upwards, and in adults (197, 200, 211, 237-246). It has transpired that early seroprevalence studies were biased by cross-reactions caused by infections with HBoV2-4, leading to over-estimations of prevalence rates for HBoV1 (237, 238). In competed antibody assays correcting for the cross reactions, a better picture of seroprevalence rates for HBoV1 has been obtained. Seroprevalences around 20% at one year of age, 50% at two years of age, 70% at three years of age and, from then on, only slight increases have been documented by this approach in Finland. Some waning of immunity through adulthood seems to occur as seroprevalence rates approach 60% in Finnish adults. Somewhat lower seroprevalence rates have been found in China and Pakistan with competed assays (238, 246). The median age for HBoV1 IgG seroconversion has been estimated to 1, 3 to 2, 3 years (211, 237, 238). However, serological diagnosis is made difficult during the first 6 to 12 months of age by the presence of maternal antibodies. Presence of maternal IgG may reduce the child’s own antibody production, leading to low occurrence of antibodies after the maternal antibodies have disappeared. This may give a bias towards higher median ages in the serological analyses. Cf. the median age of 11 months for the first PCR detection of HBoV1, measured by Martin et al (152). Nevertheless, all available evidence points to primary HBoV1 infection as being a disease of early childhood.

1.13.2.2 HBoV1 infections in immunosuppressed patients

The role of HBoV1 infections in immunosuppressed children and adults is still largely unknown. Only a few smaller studies and case reports have been published. Prolonged shedding has been reported in immunosuppressed children (247-249), and in one study prolonged viremia (presence of virus in blood) for up to four weeks was demonstrated (250). Prolonged shedding is expected in immunosuppressed children, but it has still not been

confirmed that immunosuppressed children shed HBoV1 for a longer period than immunocompetent ones. In a material consisting of children with cancer, a significant association between HBoV1 and LRTI was found, but a comparison with children without cancer was not performed (251). In immunosuppressed adults, HBoV1 seems to be rare (252), but severe cases probably caused by HBoV1 have been reported: One in a patient with chronic lymphocytic leukemia and hypogammaglobulinemia ending in multi-organ failure and death (253), and another in a lymphoma patient who contracted severe pneumonia (254). Severe development during HBoV1 infection has also been documented in a two-month old boy with hereditary lymphopenia who developed acute hepatitis (255).

1.13.2.3 HBoV1 detections in healthy children

As mentioned in the general introduction, HBoV1 DNA is often detected in nasopharyngeal samples months after a primary infection. The first thorough demonstration of this was performed by von Linstow et al in 2008 (150). They showed that HBoV1 DNA could be detectable in nasopharyngeal samples for up to five months. Shedding of this magnitude has later been confirmed by others (151, 233, 256-260) and, in a recent study by Martin et al, HBoV1 DNA shedding was documented for up to one year after the primary infection (152). This was a community-based follow-up study of healthy children. Oral fluid samples were obtained weekly to monthly during the first two years of life. The researchers also found evidence for both reactivations and reinfections of HBoV1, with periods between detections lasting up to one year. These findings have given substantial support to the idea that HBoV1 can cause persistent or latent infections. Additional support comes from studies on tonsil tissue from patients with chronic tonsillitis where occurrences of HBoV1 have been very high (30-56%) (24, 261, 262). The discovery of HBoV1 episomes, mentioned in the previous section, is also intriguing; as episomes are related to latency for herpes viruses. However, the roles of the episomes in HBoV1 infection are still undetermined. In conclusion, HBoV1 persistence and latency are both probable scenarios, but more research is needed before the issue can be settled. However, from a practical diagnostic point of view, it is clear that long-term shedding of HBoV1 makes diagnosing acute primary HBoV1 infections with DNA PCR difficult. HBoV1 DNA in a clinical sample may well represent a primary infection experienced months previously.

As HBoV1 DNA can be detected for up to a year after an acute infection, it is no surprise that HBoV1 detected by PCR is common among healthy children. This has been documented a

number of times (151, 152, 227, 228). Presence of HBoV1 in NPA from healthy children may also be explained by infections in the incubation period or by primary infections with unrecognized mild symptoms or no symptoms at all. Martin et al found that no symptoms were recorded in 38% of primary HBoV1 detections in children. This fraction was reduced to 17% if viral load was higher than 10^6 copies/ml in the NPA. In this study, samples were analyzed weekly during respiratory symptomatic events and monthly in symptomless phases. With this sampling frequency, the likelihood of missing symptoms between samplings was low. Byington et al used a similar approach, but with weekly samples taken continuously during one year and found that HBoV1 infections were asymptomatic in 54% of the cases. However, they did not make the important distinction between primary and secondary detections (233). As secondary infections are usually asymptomatic, this will lead to higher estimates of asymptomatic infections (152, 243). Nevertheless, asymptomatic primary infections seem to be common in children, and are an equally likely explanation for HBoV1 detections in asymptomatic children as prolonged shedding. Viral load is generally lower in asymptomatic than symptomatic children and might be a criterion for separating active symptomatic infections from asymptomatic infections or mere shedding of virus (152, 154, 159). The presence of HBoV1 in asymptomatic children varies considerably between studies. The difference can be striking with fractions varying between almost 50% and zero (151, 159, 220, 223, 226-228). This may be due to differences in assay sensitivity or study design. The selection of control groups is very important. It is now well known that tonsil tissue from patients with chronic tonsillitis is often positive for HBoV1 DNA. In some studies, patients admitted to elective surgery for ear-nose-throat conditions have been used as controls (228). This may lead to overestimations of HBoV1 occurrence in the control group. Other studies have used children significantly older than the patient group as controls (219). The occurrence of HBoV1 decreases with age, and this may lead to underestimations in the control groups. Another important issue is sampling methods. In a number of studies, samples from patient and control groups have been obtained with different methods. NPAs are routinely used for children admitted to hospital for RTIs. This is, however, an unpleasant procedure, which it is hard to convince healthy children to go through. Consequently, swabs are generally used for the control groups. With swabs, less secretion is obtained than with NPAs, leading to reduced test sensitivity and underestimations.

I find the presence of HBoV1 in tonsil tissue especially interesting. High frequencies of HBoV1 detections have been made in such tissue from children with chronic tonsillitis (262).

High occurrences have also been documented in sinus mucosa in patients with chronic sinusitis (263). These sites are natural targets for further studies on possible persistence or latency of HBoV1.

1.13.2.4 HBoV1 and polymicrobial infections

Children lower than two years of age are very frequently infected with respiratory viruses – 6 to 10 times per year have been estimated, as mentioned in the introduction. Given the prolonged shedding of HBoV1, it is expected that the virus will frequently be detected together with other viruses. This has been a striking finding that has received considerable attention since the discovery of the virus. In many studies, including our study described in paper II, HBoV1 has been found to be the respiratory virus most frequently co-detected with other viruses in NPAs from children (15, 26, 28, 157, 166). For this reason, it has been questioned whether HBoV1 plays any role at all in respiratory tract infections. The frequent co-detections in children with RTIs and the frequent HBoV1 detections among healthy children are strongly related phenomena. Long-term shedding and asymptomatic infections can explain both phenomena. However, a question which deserves special attention when poly-microbial infections are discussed is whether reactivation of HBoV1 is induced during respiratory infections with other viruses. This question has, however, not been thoroughly studied as yet. An additional question of equal importance is whether HBoV1 contributes to the symptomatology when it is co-detected with other respiratory viruses. Do HBoV1 and other viruses interact? As I discussed in the general introduction, studies on such interactions are demanding. First of all, large patient materials are needed to get enough samples with the virus combinations in question. In addition, the range of diagnostic tests, and their sensitivities must be comparable from study to study. HRV tests, for example, vary considerably between laboratories. This may lead to biases. A few studies addressing the interaction question for HBoV1 have been published. In some studies, all co-detected viruses have been put together in one group in order to increase statistical power, but this approach is too inaccurate and these studies will not be discussed here. This primarily leaves us with two Finnish studies from 2009 and 2014 (211, 264). Our study from 2010 will be discussed later (paper II). Söderlund-Venermo et al studied specific interactions between HBoV1, HRV and HRSV, comparing single infections with each of the combinations. They found no effects on clinical parameters or severity scores. The numbers of the specific combinations were low though, reducing the statistical power, and it is also worth mentioning that diagnosis of HBoV1 was based on serology whereas HRV was diagnosed by PCR. This creates potential

biases. Lukkarinen et al published a very interesting paper in 2014 focusing on interactions between HBoV1 and HRV. They measured cytokine profiles and occurrence of asthma after two years in included children at the age of three months to three years. HRV is a well-known risk factor for asthma development and they wanted to study whether HBoV1 had a similar effect, or whether it could influence the effect of HRV. Importantly, diagnosis was based on serology for both agents. They found that HRV was a stronger stimulant of cytokines involved in asthma development, and was expectedly more strongly associated with asthma after two years. The most interesting finding, however, was that when HBoV1 was co-detected, these effects were reduced to the level of HBoV1. This is the best evidence so far for interactions between HBoV1 and other viruses, showing, in this context, a protective effect of HBoV1. Two PCR-based studies focusing on specific virus combinations have also been published (159, 265). In both studies, the number of patients with combined infections was low, and no significant effects on severity parameters were found. One study on interactions between HBoV1 and bacteria was published by Ruohola et al in 2012 (266). They found a slightly positive correlation between HBoV1 and acute otitis media (AOM) in children whose nasopharyngeal samples were positive for pneumococci, *Haemophilus influenzae* or *Moraxella catarrhalis* (p=0.029). The association was weak, however, and larger studies addressing this question are warranted.

A related question asked by many clinicians is whether the detection of HBoV1 alone, as monodetection, can be used as a diagnostic criterion. It would be reasonable to think that when no other infecting agent is found, HBoV1 is the probable cause. This question is furthermore related to HBoV1 concentration (viral load). There is a clear association between high HBoV1 load and HBoV1 monodetection. This has been documented in many studies, including our own, as described in papers I and II (219, 258, 267). During the acute phase of the infection, when viral load is at its highest, co-detection of other viruses is less likely than later during the shedding phase, which can last for a long time. It would be expected then that HBoV1 monodetection is related to RTIs in children, and in paper II of this thesis we have documented such an association. However, the association was weak with a p-value close to 0, 05. It can hardly be used as a diagnostic criterion, because monodetection occurred in 10% of controls, and co-detections occurred in 70% of the patients.

1.13.3 Clinical associations

The first few years after the discovery of HBoV1, a considerable number of clinical and epidemiological HBoV1 papers on respiratory tract infections in children were published (74, 93, 143, 150, 159, 219-222, 224, 228, 229, 234, 235, 258, 268-281). The majority were retrospective cross-sectional studies on occurrences of HBoV1 in stored clinical hospital materials. Descriptions of clinical manifestations were given, albeit mainly without control groups for comparison. Most studies were based on immunofluorescence techniques for diagnosing other respiratory viruses. These had low sensitivities making it difficult to control for co-detected viruses. In addition, studies based on PCR for all viruses often had a narrow spectrum of viruses tested, usually lacking tests for HRVs – the most common of respiratory viruses. Symptoms described might therefore well have been caused by other viruses. Moreover, most studies were hospital based, thereby presenting a selected material consisting of the most seriously ill children. Hospital materials are also strongly influenced by local traditions for hospital admittance. Expectedly, clinical manifestations described in these early studies varied considerably. Symptoms reported in the majority of patients in most studies were coughing and rhinorrhea followed by wheezing/dyspnea and fever. In one of the most highly regarded studies from this time period, a community-based cohort study from Denmark, the frequencies were: Cough 77%, rhinorrhea 90%, dyspnoe 20% and fever 43%. General malaise was also reported in 47%, conjunctivitis in 27%, rashes in 20%, diarrhea in 23% and vomiting in 20% of the patients (150). The diagnosis was based on HBoV1 monodetection, without a distinction between primary and secondary infections. In the majority of the hospital-based materials, at least three quarters of the HBoV1 monodetection cases were LRTIs, dominated by bronchiolitis, obstructive bronchitis or asthma exacerbations closely followed by pneumonia. More accurate estimations cannot be given based on these early hospital-based studies.

Ten years after the discovery of HBoV1, a handful of community-based follow-up studies have been published (150-152, 233, 237, 243). Published in 2015, the study by Martin et al is the most thorough. They followed 87 children from birth and through to their second year of age. Samples were obtained monthly throughout the entire period, and the frequency was increased to weekly during RTIs. The children's parents were asked to fill in a standardized symptom diary during each RTI episode. This gave a good basis for detecting primary HBoV1 infections with standard HBoV1 DNA PCR, and most clinical analyses were performed solely on these infections. Symptoms registered were: Rhinorrhea 47%, cough

44%, fussiness 38%, fever 26%, rashes 7%, diarrhea 6% and vomiting 5%. To date, this is probably the best representation of HBoV1 symptomatology in children published. One weakness of the study was that dyspnea and wheezing were not registered.

Clinical biochemical or hematological laboratory parameters have usually been reported as normal in HBoV1 infected children, but leukocyte numbers and CRP are occasionally slightly elevated (159, 211, 220, 229, 234, 235, 265, 271, 275, 278).

Chest radiography has been performed in some of the hospital-based studies. Peribronchial or interstitial infiltrates are the most common findings, demonstrated in up to 70% of the children (219, 220, 271). One study by Don et al deserves special attention because it used highly specific serodiagnosis as a diagnostic criterion. Children hospitalized for pneumonia were included, but wheezing children were excluded. In this selected material, 75% of the HBoV1 infected children had interstitial infiltrates on chest radiograms (279). Hyperinflation has also been reported in considerable fractions of patients (265, 282). Lobar or bronchopulmonary infiltrates occur rarely.

Comparison with related, well-characterized infections is a very useful strategy when describing newly discovered infections. HRSV has been used as the basis for comparison in a few studies (211, 228, 229, 265). For severity parameters such as duration of hospitalization, lengths of stay in intensive care units and need for oxygen therapy, the two infections have shown similar results. However, accumulating evidence indicates that the frequency of bronchiolitis is higher in HRSV-infected children while asthma exacerbation is more frequent in HBoV1 infected children (157, 228, 265, 283). However, this difference may partly be due to age differences between the groups. The HBoV1 infected children are significantly older than the HRSV-infected. It is well known that the frequency of bronchiolitis decreases and that of asthma increases with increasing age. In one study, evidence for a higher frequency of AOM among HRSV-infected than among HBoV1 infected was found (228). The numbers of included children in the studies have usually been small, and larger clinical studies are consequently needed.

AOM is a common complication to viral respiratory tract infections, and it is consequently expected to be associated with HBoV1 infections. Frequencies varying from zero to 47% have been reported (211, 219, 228, 235, 243, 265, 280, 282). Two Finnish studies based on serological diagnosis are of special interest. In primary HBoV1 infections they found AOM frequencies to be 33% and 47%, indicating that AOM is a common complication of primary

acute HBoV1 infections (211, 243). In addition, presence of HBoV1 in middle ear fluid from patients with AOM has been documented (284-286).

HBoV1 has been detected in feces from children with diarrhea and vomiting in a number of studies (287-291). The detections have usually been made in children with accompanying respiratory tract infections, and it has been discussed whether HBoV1 actually infects the gastrointestinal tract or is just passively passing through after having been swallowed (292). It is well known that infections leading to fever often cause abdominal or gastrointestinal discomfort in children without infecting the gastrointestinal tract directly. The symptoms are probably a reaction to a systemic response to infection, an acute phase reaction. This may explain the gastrointestinal symptoms seen in some of the HBoV1 infected children. In studies with control groups, no significant associations between HBoV1 detection in feces and gastroenteritis have been demonstrated (192, 292-294).

A few case reports describing severe RTI in HBoV1 infected children have been published (295-301). Together, they describe 14 children with life-threatening LRTIs in need of intensive respiratory support; five of whom were born prematurely. Of the nine children born at term, two were reported to have underlying pulmonary disease and seven were presumed previously healthy. Reports on severe HBoV1 infections in immunosuppressed children indicate that a wider spectrum of symptoms occurs in this patient group. Severe LRTIs are frequently reported. In addition, hepatitis has been described, and severe gastroenteritis seems to be more prominent as compared to previously healthy children (247, 249, 255, 302).

HBoV1 DNA has been detected in cerebrospinal fluid (CSF) from a few children with encephalitis or encephalopathy (303-305). However, strong support for a causal link between HBoV1 and encephalitis has not yet been found. The causality question is perhaps even more difficult to answer for this patient group, where non-infectious, autoimmune encephalitides constitute a considerable proportion of the cases. HBoV1 might be just a bystander to non-infectious diseases, either by coincidence or because the virus is reactivated during inflammatory processes. The presence of a virus in CSF might be a result of a damaged blood-brain barrier due to other causes. It is interesting to note, however, that HBoV1 IgM and/or IgG were detected in CSF from three patients in one of the studies, but a comparison with serum antibody levels was not performed (305). Consequently, an evaluation of whether the antibodies had passed the blood-brain barrier passively or had been produced locally in

the brain could not be given. This is an important question when evaluating causation of intrathecal infections. A local and specific immune response has to be demonstrated.

An association between HBoV1 and Kawasaki's disease, a condition characterized by serious inflammation of soft tissue and large blood vessels, has also been suggested by some authors (306, 307). However, the results have been conflicting (306-310). As so often with Kawasaki's disease, it has proven difficult to establish a relation with a specific infectious agent. The search continues.

This concludes my overview of current knowledge about HBoV1. In the following I will go through the three papers included and end the thesis by discussing the causal link between HBoV1 and respiratory tract infections.

2 Aims of the study

2.1 Principal objective:

The main aim of the project was to contribute in clarifying the causal role of HBoV1 in respiratory tract infections in children

2.2 Specific objectives:

- To investigate associations between HBoV1 concentrations in respiratory samples and respiratory tract infections in children
- To investigate associations between presence of HBoV1 in blood and respiratory tract infections in children
- To search for genetic features in HBoV1 associated with LRTI and URTI.

The last aim was a generally formulated aim at the start of the project. HBoV1 mRNA became the primary genetic feature focused on during the project period.

3 Material and methods

3.1 Study area

The study was performed at St. Olav's Hospital in Trondheim, Norway, a tertiary hospital for Mid-Norway encompassing a population of 711 000. It is the local hospital for the County of Sør-Trøndelag with a total population of 311 000, of whom 59 000 are children below 16 years of age. More than half of the county's population, 185 000 inhabitants, live in the city of Trondheim.

3.2 The Childhood Airway Infection Research project

The project "Respiratory tract infections in children", later renamed "Childhood Airway Infection Research" (CAIR), was started in 2006. A database was established for clinical and microbiological information gathered from children admitted for RTI to the Department of Pediatrics in our hospital. The aim was to include all children admitted for RTI from November 2006 onwards. At the same time, a biobank containing all sample material collected from the included children was established. From June 2007, a parallel cohort of healthy children served as a control group. Those were children admitted for elective surgery and who had not had symptoms of RTI during the last two weeks. A total of 628 children had been included by June 2015. Most of them were admitted for cryptorchidism, hernia repair and skin tumors. None were admitted for ear, nose and throat surgery.

All three papers in the thesis are based on the CAIR material. The numbers of samples included in each study increased successively as the size of the cohort material increased. By June 2015, 3285 patients and 628 controls had been fully included after informed consent. Altogether, a total of 7201 patient samples was collected, including 3916 samples with incomplete clinical data sets.

The CAIR-study is today organized by the CAIR group, a collaboration between Department of Medical Microbiology and Department of Pediatrics at St. Olav's Hospital, and the Department of Laboratory Medicine, Children's and Women's Health at the Norwegian University of Science and Technology (NTNU).

3.3 Clinical information

Clinical data were collected prospectively by the use of standardized forms, filled in by the treating consultant pediatrician or registrar. Due to administrative and staffing issues, plotting of data into the database was subject to some delay, and variables containing clinical data were not completed until recently. Therefore, a large part of the clinical information in my three articles had to be obtained from electronical hospital records. Since the clinical information in the hospital records was not always complete to fit into the detailed categories in the registration form, we decided to use only two crude clinical categories: LRTI and URTI. LRTI was diagnosed in the presence of dyspnea, signs of lower airway obstruction (wheezing or retractions) and/or a chest radiogram showing infiltrates, atelectasis and/or air trapping. URTI was diagnosed when rhinitis, pharyngitis and/or AOM were registered without signs of LRTI.

3.4 Microbiological diagnosis

In all cases where parental consent was given, NPA was obtained from the child. If possible, EDTA-blood was also drawn for storage of plasma. NPAs from controls were obtained during general anesthesia for elective surgery.

In papers I and II, HBoV1 diagnosis was based on detection of HBoV1 DNA by real-time PCR. A qualitative PCR was used in paper I, and semi-quantitative evaluation was performed based on Ct-values. Paper II was also based on semi-quantitative evaluations, but a quantitative PCR was established and used for defining cut-off values. In paper III, I describe an HBoV1 mRNA PCR which was established and added to the diagnostic panel. I will describe this test in detail below. The target for the HBoV1 DNA PCR was the NP-1 gene. Primers and probe are described in paper I. The quantitative PCR (papers II and III) was established by use of a cloned plasmid containing the PCR product. Concentration was measured by light absorption, and serial dilutions covering a range of seven logs were made to create a standard curve.

All NPAs collected in the CAIR study were tested for HBoV1 with the same HBoV1 DNA PCR during the whole project period. In addition, they were tested for HAdV, coronavirus (OC43, 229E and NL63), enterovirus, HMPV, influenza A and B virus, parainfluenza virus type 1–3, RS-virus (RSV), HRV , *Bordetella pertussis*, *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*. In paper III, PCRs for parainfluenza virus type 4 and parechovirus

were also included. All tests were in-house real-time PCRs based on TaqMan probes. The analyses were carried out as part of the daily laboratory routine and generally performed within 24 hours after sample collection. Plasma samples from children positive for HBoV1 DNA were, when available, also tested with the HBoV1 DNA PCR. Presence of HBoV1 DNA in the plasma samples was used as a marker for viremia (papers II and III).

The virus transport medium used for the NPA samples did not contain antibiotics. Antibiotics were avoided in order to enable bacterial detection by culture on standard agarose media. In addition, all samples were cultured for viruses using standard cell lines. HBoV1 is not culturable in these cell lines and the latter procedure was consequently of little relevance for my thesis.

A semiquantitative approach based on Ct-values was used in all three papers, but was supplemented with exact quantitation for HBoV1 in papers II and III. In paper I, semiquantitation was the sole quantitative approach and categories high, medium and low viral load were used. Cut-off values were set to Ct 28 and Ct 35. In addition, the term "dominating virus" was introduced and solely used in this paper when the Ct-value for HBoV1 was at least three cycles lower than for any other virus in the same sample, corresponding to a concentration ten times higher. This approach is a rough approximation because it is based on a comparison of different PCRs targeting different agents. The PCRs may have different effectivity scores, although they all showed good results in our laboratory's internal evaluation. Exact quantitation of all PCR results would be a necessary next step along this line of thought. This was not prioritized at the time, and the term "dominating virus" was therefore not used in papers II and III. The term was introduced in paper I to add momentum to the data on HBoV1 monodetection. The hypothesis was that, when HBoV1 was present in a significantly higher amount than any other virus, this would indicate clinical significance, corresponding to detecting HBoV1 alone.

HBoV1-specific mRNA was used as an alternative HBoV1-specific target in paper III. Parvovirus mRNA is produced by host cell RNA-polymerase II. Subsequent mRNA processing follows host cell pathways and mRNA splicing (alternative recombining of mRNAs) is an important part of this. By use of an in-vitro model based on recombinant HBoV1, Chen et al were able to map the transcription profile of HBoV1 in 2010 (figure 4) (210). Their work confirmed the results of a previous study by Dijkman et al with only small modifications (206). My choices of primers and probe were based on these studies. In figure 6

the positions of primers and probe are illustrated. The primers target the segments at positions 203-223 (forward) in a non-coding region upstream of the gene NS1 and 2438-2418 (reverse) in a regulatory region just upstream of the gene NP1. In the HBoV1 genome the distance between the primers is so long (2236 bases) that PCR-amplification will not happen under normal conditions. Amplification of genomic HBoV1 DNA should consequently not occur with these primers. Through reverse transcription, mRNAs were converted to cDNA. According to figure 4, the primers should be considerably closer together on some of these cDNAs. Theoretically, two products of 242 and 363 base pairs should be produced.

Total DNA and RNA were extracted by using NucliSens easyMag automated extractor (bioMerieux, Marcy l'Etoile, France). Reverse transcription was carried out with random primers and reverse transcriptase at 37°C for 60 min. followed by 94°C for 10 min. The PCR was first performed at 45 cycles at 95°C for 5 s., 55°C for 10 s. and 72°C for 20 s. This is the temperature profile published in paper III. After additional optimization we changed to a two-step profile of 95°C for 5 s. and 55°C for 30 s. in 2014.

Every sample was also run without reverse transcription (non-RT-controls) to test for reactions with viral genomic DNA, as this could be a source of false positive reactions. For a detailed description of all controls used, see paper III.

A recurring problem when working with RNA is low stability of the molecules when exposed to extracellular environments. RNAse enzymes are present in any organism as well as on skin flakes and hairs and are consequently ubiquitous in human samples and in a laboratory environment. RNAs are usually degraded within minutes when exposed to RNAses. For this reason, it was important to examine the stability of HBoV1 mRNAs in clinical NPA samples. This was done by comparing samples stored from zero to five days at room temperature and at 4°C. Ct-values obtained in the same run were compared. RNAse-free precautions were followed in the laboratory by use of gloves, gowns, RNAse-free pipettes, tubes and buffers, and safety cabinets.

Quantitative standards for the HBoV1 mRNA PCR were made by cloning a plasmid containing the PCR product of 242 bp. The amount of nucleic acid was measured, and serial dilutions covering a range of seven logs were made to measure the analytical sensitivity of the HBoV1 mRNA PCR. Evaluation of the reverse transcription step was not included with this method. It had to be measured separately by making serial dilutions both before and after

reverse transcription. Results around 100% for both steps indicate minimal loss of effectivity in the reverse transcription step.

The specificity of the mRNA PCR was evaluated by testing cDNA from samples positive for 18 different respiratory viruses and bacteria, four viruses in the herpes virus group known to reactivate in respiratory secretions, and three human viruses within the *Parvovirinae* subfamily: parvovirus B19, HBoV2 and HBoV3 (for details see paper III).

3.5 Statistical analysis

Statistical analysis was by Chi-squared test for categorical variables and Student's t-test for continuous variables. Multiple logistic regression analysis was used to evaluate the association between HBoV1 and respiratory tract infection. RTI (children with RTI versus controls) was used as dependent variable, and HBoV1 as independent variable. In addition, age, gender and the presence of other viruses were included as independent variables in an effort to control for differences in these variables among cases and controls. I report the odds ratio (OR) with 95% confidence interval (95% CI) and the corresponding p-value as a measure of the strength of the association. All analyses were performed using IBM SPSS Statistics software.

3.6 Ethical approval

The study was approved by the Regional Committee for Medical and Health Research Ethics in Mid-Norway, and the CAIR biobank was approved by the National Biobank Council.

4 Results

4.1 Paper I

This paper describes a clinical pilot study. It was based on data from the first four months of the CAIR project, from November 2006 to March 2007. In total, 376 NPA samples from children with RTI were examined for 16 infectious agents in addition to HBoV1, all with real-time PCR. Viral and bacterial culture was also undertaken. The aim was to study the epidemiology of HBoV1 in our material, and to evaluate the clinical impact of semiquantitative PCR-results.
Of these, 45 of the 376 samples (12%) were positive for HBoV1. No seasonal changes in occurrence of HBoV1 were observed during this short period, in contrast to HRSV, coronavirus OC43 and influenza A-virus which showed significant variations. The median age of patients was 17 months, and 58% were boys. In 78% of the HBoV1-positive samples at least one other virus was detected (Tab. 3). HBoV1 was the virus most frequently co-detected with other viruses, but co-detections were nearly as common in HAdV and enterovirus infections. Moreover, among patients with HBoV1 monodetection, 41% had a high viral load and only 4% had medium or low viral load ($p<0,001$, Fisher's exact test). 71% of the patients had LRTI (of which 62% had bronchiolitis and 38% had pneumonia), and 20% had URTI. Eighty-eight percent of patients with HBoV1 recorded as the only or dominating virus, and 50% of the other patients, had LRTI ($p=0,005$, Fishers exact test). However, no significant associations between viral load and RTI diagnosis were found.

This paper was written by invitation for a special issue of Journal of Clinical Virology dedicated to respiratory viruses. The invitation came after presentation of the data on a congress arranged by the European Society for Clinical Virology (ESCV) on respiratory viruses in Lyon, may 25. 2007.

Table 3. Number of co-detected viruses in 35 samples positive for HBoV1 DNA. The number of detections made by PCR and viral culture are shown. Right column: Number of samples with at least two viruses detected in addition to HBoV1.

	Number of detections			Part of triple/quadruple infections
	Total	PCR	Culture	
HAdV	6	5	3	4
Coronavirus OC43	10	10	-	6
Coronavirus NL63	1	1	-	-
Enterovirus	9	9	1	8
Human metapneumovirus	7	7	1	4
Influenza virus A	3	3	3	3
Parainfluenzavirus type 3	5	5	2	3
Rhinovirus	10	10	-	8
RS-virus	8	8	7	3

4.2 Paper II

The study described in paper II was an expansion of the initial study. The number of included patients (n=1154) was considerably increased in order to enhance statistical power. In addition, a control group (n=162) was included, enabling us to study the relationship between HBoV1 and RTI more closely. NPA samples collected over a two-year period from June 2007 to August 2009 were examined for HBoV1 and 16 other respiratory agents. The same test panel as described in paper I was used including viral and bacterial culture.

One-hundred and forty four of 1316 samples (11%) were positive for HBoV1. Seasonal variations in HBoV1 occurrence were observable, but when we adjusted for total number of samples received per month the variations in occurrence were not significant (Fig. 5). Average age of patients was 19 months (median 17 months), and of controls 33 months (median 31 months). Sixty-three percent of the patients had LRTI (of which 49% had bronchiolitis and 14% pneumonia), and 37% had URTI. HBoV1 was detected in 10% of patients and 17% of controls. When adjusted for age, gender and presence of other viruses HBoV1 was not associated with RTI in children (OR: 0.8, 95% CI: 0.5–1.3, p= 0.30). In 75% of the HBov1-positive samples at least one other virus was detected. HBoV1 was the sole virus in 29% of the patients and in 7% of the controls (p=0.02). The association persisted after adjusting for age and gender in a logistic regression analysis (OR: 5.2, 95% CI: 1.1–24.4, p= 0.04). A high viral load (>10^6 copies/ml) was not associated with RTI, but interestingly an even higher viral load of >10^8 copies/ml was found in 14 patients and none of the controls. Additionally, a high viral load (>10^6 copies/ml) was associated with LRTI, also when adjusted for age, gender and other viruses (OR 3.6, 95% CI: 1.2-10.7, p=0.02). Viremia on the other hand, was clearly associated with RTI. It was found in 18/40 (45%) of patients and in none of the controls. In addition, 16/28 (57%) the LRTI patients and 2/10 (20%) of URTI patients had viremia (p=0.02). One plasma sample was examined with an electron microscope showing particles compatible with parvoviruses. HAdVs and enteroviruses were significantly more frequent in HBoV1-positive samples than in HBoV1-negative samples, but co-presence of these viruses in any combination was not associated with an increased frequency of RTI. No interaction between HBoV1 and bacteria were observed.

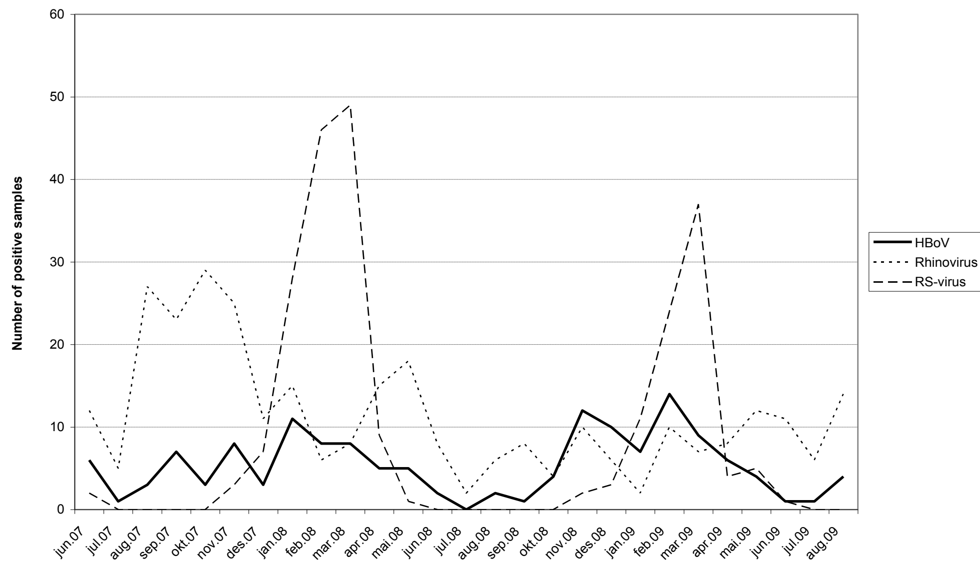

Figure 5. Monthly distribution of samples positive for HBoV1, HRV (rhinovirus) and HRSV (RS-virus), in the period June 2007 - August 2009.

4.3 Paper III

Paper III describes a new principle for detecting respiratory viruses. It is based on detection of virus-specific mRNA which theoretically would imply specific detection of actively transcribing viruses. The development of an HBoV1-specific mRNA test and its validation is described. Validation was carried out on clinical material consisting of 161 HBoV1-positive NPA samples, collected through the CAIR project over a three-year period (from June 2007 to June 2010). Of these, 133 were from children with RTI, and 28 were from controls.

Thirty-three of the samples were positive for HBoV1 mRNA. As mentioned under Material and Methods, two product sizes of 252 and 363 base pairs were expected after theoretical considerations. Gel electrophoresis showed bands fitting well with this prediction, and sequence analyses confirming the results are illustrated in figure 6. Products with either one or two segments (introns) removed were created.

Thirty of the 33 HBoV1 mRNA-positive samples were negative for the non-RT controls. As mentioned above, these samples were included to control for possible false positive reactions with viral genomic DNA. The remaining three samples gave weak signals, and additional analyses were required. All three samples had very high HBoV1 loads, and showed strong mRNA signals after cDNA synthesis. The PCR products from the reactive non-RT controls were then sequenced. Product sizes were 145, 261 and 457 bp, showing gaps at apparently random positions, all lacking splice site characteristics. Amplification efficiency was calculated based on dilution series both before and after cDNA synthesis. This was done to evaluate the effectivity of both cDNA synthesis and the final PCR. Both results were 100%, indicating minimal loss of efficiency in both steps of the RT-PCR. The PCRs detection limit for the plasmid containing the PCR-product was 500 copies/ml (10 copies/reaction). To ensure that mRNA had not been degraded during storage, we used an RT-PCR specific for the human gene β-actin. This gene is universally and stably expressed in humans and is a common control in gene expression studies. It was positive for all samples. Analytic specificity was tested by running the HBoV1 mRNA PCR on cDNA from samples positive for 25 other viruses and bacteria commonly present in the respiratory tract. All samples were negative. Tests on mRNA stability in the NPAs showed that the target mRNA was stable at 4°C for five days, but was significantly reduced after three days at room temperature.

Thirty-three of the 133 patients and none of the 28 controls were positive for HBoV1 mRNA. Twenty-seven of 86 children (31%) with LRTI and 6 of the 47 children (13%) with URTI

were positive for HBoV1 mRNA. The difference remained significant after adjustment for age, gender and presence of other viruses (OR 3.5, 95% CI: 1.3-9.8, p=0.02). Expectedly, significant associations were also found for HBoV1 viremia and HBoV1 monodetection, as these factors have previously been found to correlate with RTI in children. In addition, a strong association was found between HBoV1 mRNA and HBoV1 DNA load.

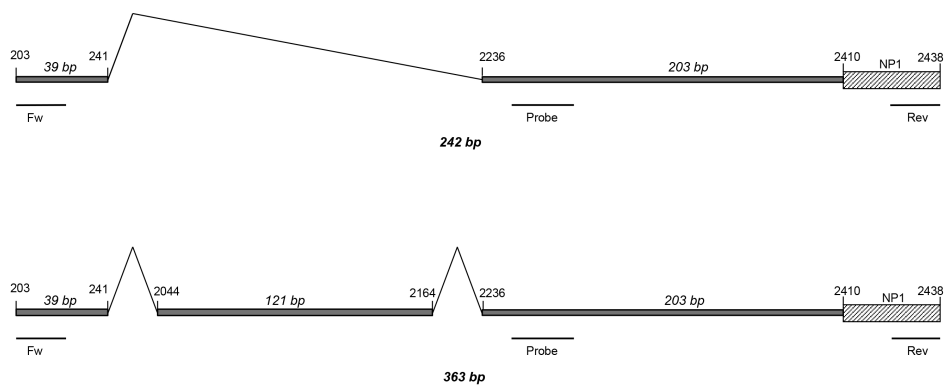

Figure 6. Schematic representation of the two HBoV1 mRNA PCR products, illustrating alternative splicing. Positions of primers and probe are shown. The total length of the upper product is 242 base pairs and of the lower 363 base pairs (Reference sequence: GenBank accession no. NC007455).

5 General discussion

The main aim of this work was to contribute in clarifying the causal role of HBoV1 in RTIs in children. First, a pilot study was conducted providing preliminary epidemiological data (paper I). A larger controlled clinical study (paper II) was established to test hypotheses gained in the first study. In paper II, substantial evidence for a causal link was documented. However, the many detections of HBoV1 DNA among healthy children posed a problem. If a marker of actively replicating viruses would correlate better with RTI than HBoV1 DNA, this would give additional support to the hypothesis of a causal link. We chose HBoV1 mRNA as a candidate target, as mRNA is a marker of viral transcription and by this an indirect marker of viral replication. This led to paper III, which indeed gave support to the hypothesis that HBoV1 causes RTI in children. I will now discuss my findings in relation to the main objective of this thesis - whether HBoV1 is causally linked to RTI in children.

5.1 Demographic data

Papers I and II demonstrate that it is predominantly children younger than two years of age that are infected with HBoV1. The median age was found to be 17 months. If we focus exclusively on children with acute infection and viremia or a positive HBoV1 mRNA test, the median ages were found to be 14 months for both. A thorough follow-up study performed in the USA found median age for the first HBoV1 infection in children to be even lower (11 months) (152). Papers I and II also demonstrate that boys are infected with HBoV1 more frequently than girls. Both the age and gender distributions are in accordance with what we see for other respiratory viruses which are well established as causes of RTI in children. Similarities in the epidemiological profile between HBoV1 and other respiratory viruses are not strong arguments for causality, but fit well with the hypothesis and encourage further studies. However, the epidemiological profile of HBoV1 differs considerably from most other respiratory viruses in one aspect. No significant seasonal variations in HBoV1 occurrence were observed in our studies. Most other respiratory viruses show considerable variations throughout the year, and some are causing large outbreaks. No such pattern was observed for HBoV1, which seems to occur sporadically all year round. This has been observed by others (152, 277). An important factor might be the viral genome. Most respiratory viruses are highly variable RNA viruses, while HBoV1 is a more stable DNA virus (see section 1.1.2). The only other DNA virus among the respiratory viruses, HAdV, behaves similarly to HBoV1. Respiratory DNA viruses seem to have a different spreading pattern compared to the

RNA viruses. The observed pattern is consequently not an argument against a causal role for HBoV1 in RTI in children.

5.2 Case observations

Direct observation of spread of HBoV1 infections in a transparent and partly closed environment such as a hospital ward may be used as an argument for causality, when one observes the development of respiratory symptoms in patients who were observed not to have such symptoms before the event. If HBoV1, and no other virus, is detected in each affected patient this indicates a pathogenic role of the virus. This has been documented a number of times (220, 225, 277, 295, 311, 312). In some of the reports the epidemiological link between the patients was unclear, but Kesebir et al could in their report from 2006 demonstrate a spatial and temporal link between two patients. However, it must be noted that some of the reports were from ICUs and included children with complex conditions involving prematurity, congenital heart diseases, post-transplantation conditions and immunosuppression. The respiratory symptoms may have been caused by underlying conditions, and it cannot be ruled out that HBoV1 was simply a bystander without clinical relevance in some of the cases.

5.3 Multiple infections

The high frequency of co-detections in children with positive HBoV1 DNA tests, was early used as an argument against a causal role for HBoV1 in childhood RTIs. The term "innocent bystander" often appeared in the literature. Indeed, HBoV1 was the virus most frequently detected together with other viruses, also in our studies, but interesting patterns emerged during sub-analyses. In the first study we found that viral load was significantly higher when HBoV1 was detected alone than when it was detected together with other viruses (paper I). In other words, co-detections were less likely when viral concentration was at its peak. According to classical observations of acute respiratory virus infections, symptomatic disease coincides with a peak in viral load in NPAs. That fewer candidate pathogens were present at or around this peak was therefore an interesting observation. The association between monodetection and viral load was later confirmed using the larger study in paper II. This study included a control group, and we could also demonstrate that HBoV1 monodetection was more common in children with RTI than in controls. This gave support to the argument that when HBoV1 is detected alone the chances are higher that the detection is clinically relevant. In symptomatic children with low HBoV1 load and co-detections, HRSV was most

frequently detected. HRSV is well established as a respiratory pathogen and the most likely cause of the symptoms in these children. On the other hand, by use of better criteria for acute HBoV1 infection, such as viremia, detection of HBoV1 mRNA and serodiagnosis, we and other researchers have documented that in high fractions of children with acute HBoV1 infection other viruses are co-detected. In 71 % of viremic children (paper II), in 58 % of HBoV1 mRNA-positive children (paper III) and in 74 % of children with a serodiagnosis, other viruses are co-detected (211). These are strikingly high percentages, and it is tempting to speculate that other viruses play roles in acute HBoV1 infections and/or vice versa. We are only seeing the beginning of a research field here, on interactions not only between host and pathogen, but also between pathogens.

These analyses exemplify the complexity of RTIs in children. Different viruses infect children simultaneously or serially, sometimes causing disease and sometimes not. In addition, interactions between the infectious agents may occur, either enhancing or inhibiting each other's clinical effects. These effects complicate clinical evaluations even more. Further research is warranted, and the main conclusion I can give at this point is that the presence of multiple infections per se cannot be used as an argument against a virus' causative role.

5.4 Viral load

It is important that exact measurements on biological samples are performed on standardized sample materials. This is not easy to accomplish for NPA samples. Sample volume and quality may vary considerably from sample to sample, depending on both sampling technique and child cooperation. Technical biases may be the result. For this reason we found it appropriate to use a semi-quantitative approach with only the categories high and low viral load in our studies. We first used three categories: high, medium and low viral load, but the distinction between medium and low viral load turned out to provide limited information. The two were consequently merged in a single group, and the cut-off between high and medium/low viral load was set to 10^6 copies/ml.

Given the association we found between HBoV1 load and HBoV1 monodetection (paper I), we were surprised not to find an association between viral load and RTI in the second study with the control group (paper II). By use of Chi-squared tests, and logistic regression analyses controlling for age, gender and other viruses, we did not find significant differences regardless of where the cut off was set. With the cut-off 10^6 copies/ml, 33% (39/117) of the patients and 15% (4/27) of the controls had a high viral load. This cut-off gave the largest difference

between the groups but it was far from reaching statistical significance (OR 1.4, 95% CI: 0.4-5.1, p=0.57). It was, nevertheless, interesting to find that 14 patients and no controls had a very high viral load (>10^8 copies/ml). The main reason for the negative results in the statistical analyses was that a high number of patients had a low HBoV1 load. As mentioned, the majority of these were in addition positive for HRSV, indicating that this virus was the principal pathogen in these patients. Another possible source of bias was the differences in duration of symptoms before admission to hospital. Some children are admitted in the acute phase of the disease when a high viral load can be expected, while others are admitted several days later when viral load is expected to be decreasing. We did not control for this. Data on duration of symptoms have now been released from the records, and we will include them in future studies.

However, a high viral load was more common in children with LRTI than URTI (OR: 3.6, 95% CI: 1.2-10.7, p=0.02), suggesting that HBoV1 reproduction was more pronounced in more severe cases with RTI. This may indicate a dose-response effect. Alternatively, it tells us about selection bias. Children with LRTI may be more quickly admitted to hospital during the acute phase of the disease than children with URTI. Despite the possible influence of these biases, the facts that viral load was higher in patients with LRTI than URTI, and that more children in the patient group than in the control group had very high viral load (>10^8 copies/ml), support a causative role of HBoV1 in childhood RTI.

As discussed in the general introduction, the presence of HBoV1 in healthy controls has varied between studies, probably depending on study design. Some studies reporting its presence in healthy children have found significantly higher viral loads among patients than controls (152, 154, 159). Martin et al's study from 2015 is worth special attention as their follow-up design eliminated many confounding factors. Patients were tested as soon as possible after they experienced symptoms, and their thorough follow-up regime ensured the detection of primary acute HBoV1 infections. With this design they could compare truly acute HBoV1 infections with healthy controls. As described in section 5.8.2, they found significant associations between a high viral load and three clinical parameters. Their results supported the causality hypothesis. Moreover, this indicated that confounding factors had influenced our data on viral load.

5.5 Viremia

Presence of virus in blood (viremia) during acute infection is a common feature in viral infections. The timing and duration of viremia can vary considerably, but for respiratory viruses (HRVs, influenza A virus and enteroviruses), it is usually short term and peaks around the time the patient experiences symptoms (313-316). As discussed above, qualitative detection of HBoV1 DNA in NPA is poorly associated with RTI in children, partly due to virus shedding after the symptomatic period. Viremia is expected to have a much shorter duration, and would consequently be expected to correlate better with RTI. Indeed, our results showed a strong association with RTI, because 45% (18/40) of patients with available blood samples, and none of the controls (0/20) were positive. This finding gave substantial support to the hypothesis that HBoV1 causes RTI in children.

5.6 mRNA

Detection of HBoV1 DNA correlates poorly with RTI and the standard HBoV1 DNA PCR is consequently not suitable for clinical use. A positive test only tells us that viral genomes are present but does not give direct information about the virus' replication competence. Viral culture would have been a natural gold standard for detection of replication-competent HBoV1, if available. Cell models suitable for use in HBoV1 diagnostics are lacking today, and during the search for alternatives, the idea to test for HBoV1 mRNA was proposed. Tests for HBoV1-specific proteins were also considered, but the mRNA test soon showed promising results and I gave this my full attention for the rest of my stipend period.

Production of viral mRNA is an indirect marker of virus replication. The quantity of mRNA produced will depend on many factors. It may correlate with virus concentration, but may also vary depending on the level of expression of the target gene. For diagnostic purposes, we found it sufficient to aim for a qualitative test, only determining whether or not HBoV1 mRNA was present, thus indicating whether replicating and transcribing virus was present. Parvoviruses, as most small DNA viruses, exploit the host cell replication and transcription machinery, only adding regulating factors of their own. Splicing is a central part of eukaryotic transcription, and we decided to explore whether this principle could be used to differentiate between an active and non-active state of HBoV1 in the studies on causality. We chose an approach based on primers spanning a splice site in the HBoV1 genome. After reverse transcription, a shortened spliced segment should be amplified, and the original viral genomic DNA should not be amplified because of the large distance between the primers on this target.

The nucleic acid extracts were initially treated with DNAse to ensure that no viral genomic DNA was amplified, but this proved to be unnecessary. To control for this, we included a PCR-run on all the extracts before reverse transcription, to see if any genomic DNA was amplified. In three of 33 samples amplification was actually observed, but only weakly. Sequencing showed that the products were chimeras or recombinant DNA, and they all appeared in samples with large amounts of nucleic acids. After reverse transcription, amplification was much stronger - indicating the presence of considerably higher amounts of mRNA in these samples (Ct-values were below 30). We concluded that the small amounts of viral genomic DNA amplified in the three samples did not influence the results, as mRNA clearly dominated. The chimeras or recombinants produced were probably the results of random processes going on in samples with high concentrations of viral DNA. If future studies show stronger amplification of viral genomic DNA in some cases, the problem may be solved by using a probe spanning the splice site, ensuring amplification signals from only the reversely transcribed mRNAs. Nevertheless, we could conclude that the 33 positive samples in our material were confirmed HBoV1 mRNA-positive samples. HBoV1 mRNA was detected in 33/100 (25%) of the patient samples and in none of the controls. This clear association gave additional support to the hypothesis that there is a causal link between HBoV1 and RTI in children. In other words, actively transcribing virus was strongly associated with disease. This fitted in neatly with the findings for viremia, high viral load and monodetection. Viral load and presence of mRNA were strongly correlated, and might be said to represent the same process. The problem, however, has been that strong correlations between viral load and RTI were difficult to obtain in our data, due to confounding factors. The mRNA results from this study were the strongest arguments until then for an association between high rates of viral replication/transcription and RTI in children. They supplemented and strengthened the viral load data nicely. Whether or not the mRNA test will be the most suitable test for diagnostic use remains to be determined.

5.7 Strengths and weaknesses of papers I-III

A major strength of all three of our studies was the broad panel of real-time PCRs used, which included tests for 14-16 viruses and three bacteria. In addition, all samples were cultured for viruses and bacteria with standard cell lines and agar media, respectively. Such extensive test protocols were unusual at the time paper I was published, but broad PCR-panels have now become standard. The first study was a pilot study (paper I), and included a limited number of patients. The two later studies included samples from extensive source materials consisting of

up to 2379 patients. This was a major strength of these studies leading to high statistical power (papers II and III). Furthermore, a control group was prospectively included in the same time period and from the same population as the cases, and the same sampling technique and test algorithm was used for both cases and controls. The controls were admitted to elective surgery, and hence one might argue that they were not true healthy controls. A possible bias could, for example, be admittance to elective surgery for infection-related diseases. However, lack of respiratory symptoms during the last two weeks was an inclusion criterion for the control group, and the children were only admitted for congenital malformations such as for example hernia and undescended testicles. Importantly, none were admitted for ear-nose-throat diseases. As a consequence of the selection approach, the control group had a higher average age and a higher frequency of boys than the case group. Through a logistic regression analysis we were able to adjust for this. Another possible bias which needs consideration is under-communication of mild symptoms by parents. Presence of RTI will usually lead to a postponed operation, and some parents might be tempted to avoid such delays by not reporting minor symptoms. All children included in the control group were examined by an anesthetist before surgery. This reduced the risk for inclusion of children with obvious symptoms.

A further strength of our studies was that all PCRs were performed daily by experienced technicians in a certified diagnostic laboratory, using strict routines for avoiding contamination. All PCRs were unchanged during the study period.

An important weakness with our studies was that they all were hospital based. This created a bias towards more severe disease. The occurrence of viruses in such material may be very different from that obtained in the community, and this difference has to be kept in mind when epidemiological features are evaluated. Nevertheless, hospital-based material is well suited for studying causality, but one has to keep in mind a possible bias caused by overrepresentation of susceptible children with underlying conditions which could make them more prone to infection. Strictly speaking, we did not control for this in our studies as detailed clinical data had not yet been released. However, in order to reduce the bias, children admitted to the cancer ward were excluded from the studies described in papers II and III.

A second weakness I will emphasize is the crude classification system used for clinical conditions. We used only two categories: LRTI and URTI. For this reason, limited clinical information was gained from the studies. On the other hand, the classification led to higher

numbers of patients in each group. This gave the necessary statistical power to detect the associations reported between LRTI and viral load, viremia and presence of mRNA.

The cross-sectional design of the studies may also be a limitation because the children were only sampled once during the course of the disease. This is especially relevant for the viral load data, where the effect may be increased variability of the data. Viral load varies substantially from day to day during the course of an infection, and a follow-up design is necessary in order to get the full picture of the dynamics. This can be compensated for by controlling for duration of symptoms before admission. We did not do this in the studies presented, but such data are available in the recently released study records, and will be included in future studies.

5.8 Causality according to the Bradford Hill-criteria

I have argued that the Bradford Hill-criteria are the most suitable guidelines for discussing a causal link between HBoV1 and respiratory tract infections (see section 1.12). When good data from experimental models are lacking, I find it appropriate to use well established epidemiological guidelines. Today, eleven years after the discovery of HBoV1, available epidemiological data are in my opinion sufficiently reliable to make a compelling case in favor of a causal relationship. I will go through the nine Bradford Hill-criteria, step by step.

5.8.1 Strength of the association

As mentioned, the common presence of HBoV1 in healthy children has made association studies for this virus difficult. However, we have demonstrated associations between HBoV1 monodetection, a high viral load, and presence of HBoV1 DNA in plasma (viremia) and RTI in children (paper II). In the following I will use odds ratios (OR) when applicable as measurements of strength. For viremia, we found a very strong association. The OR was indefinite as there were no children with viremia in the control group. Moderately high adjusted ORs were found for the associations between high HBoV1 load and LRTI (OR 3.6, 95% CI: 1.2 – 10.7) and between monodetection and RTI (OR 5.2, 95% CI 1.1 – 24.4) (paper II). The strong association found between detection of HBoV1 mRNA and RTI adds to the body of evidence in this context (paper III). As for viremia, OR was indefinite because no children in the control group were HBoV1 mRNA-positive.

5.8.2 Consistency

The consistency criterion implies that the associations registered are reproducible. A few other studies based on broad PCR-panels and controls matched or adjusted for age, time and

place have shown similar associations. Fry et al compared HBoV1 frequency in children with pneumonia with an age-matched control group. They found a difference even when viral load was not accounted for. The OR was 4.39 (95% CI: 1.36 – 14.15) (159). Zhao et al collected NPAs from 554 children with bronchiolitis or pneumonia and nasal swabs from 195 healthy control children. They found an association between the mere presence of HBoV1 and RTI (OR 2.88, 95% CI 1.12 – 7.41), and an indefinite association between a high viral load ($>10^6$ copies/ml) and RTI as no controls had a high viral load (154). The American study by Martin et al from 2015 did not include tests for other viral pathogens, but it is nevertheless relevant because of the thorough follow-up regime used, ensuring detection of acute primary HBoV1 infections. Controls were included from the same cohort in asymptomatic periods, reducing age and selection biases. They found associations between detection of any quantity of HBoV1 and new cough symptoms (OR 5.6, 95% CI: 1.4 – 5.5) and visits to a healthcare provider (OR 2.8, 95% CI: 1.02 – 7.7). Moreover, detection of a high HBoV1 load ($>10^6$ copies/ml) was associated with new onset of respiratory symptoms (OR 5.6, 95% CI: 2.1 – 15.1) (152).

Two other studies, however, did not show significant associations (150, 151). Some details in study design may explain the lack of significant results. Martin et al, in their 2010 study, included controls only at enrollment and ended up with 45 controls and only 41 time-matched patients, leading to low statistical power. In addition, the study population contained a high proportion of children 0-6 months old. In this age group, maternal antibodies may reduce the occurrence of symptoms, and this may have led to underestimations of symptoms in the patient group as a whole. Linstow et al found no significant differences in viral load between the patient group and the control group. This is in line with our 2010 study (paper II), but unlike our study they did not report differences between diagnosis groups either. This may be explained by the discrepant use of clinical categories. Instead of the distinction between LRTI and URTI, they used a finer classification system. With only 57 HBoV1-positive patients included, the numbers in each clinical category was low, leading to reduced statistical power. In addition, they found no differences in the occurrences of HBoV1 monodetection, neither between patient group and control group, nor between different diagnosis groups. HBoV1 monodetection was the weakest factor associated with RTI in our study, and it may thus vary from study to study depending on methodological factors such as performance of each of the PCRs used and sampling methods. Finally, they did not test for viremia in any of the studies. This was the factor most strongly associated with RTI in our study (paper II).

5.8.3 Specificity

According to the specificity criterion the disease should be less prevalent when the infectious agent is not present. With the high occurrence of other respiratory viruses in children giving indistinguishable clinical manifestations, it is difficult to fulfill this criterion. You need to control for all other known respiratory pathogens, and compare the frequency of RTI in a group without viruses detected with an HBoV1-positive group. To avoid selection biases, the best approach would be to perform the comparison in the community and test all children in a defined environment regardless of symptoms. Frequency of symptoms in a virus negative group could then be compared with an HBoV1-positive group. Hospital based materials such as ours will usually include a high proportion of patients and a considerably lower proportion of healthy controls. This will give a bias towards disease in the total material, but a comparison can nevertheless be done between the two groups as long as the virus-negative group is large enough to give adequate statistical power. In 7201 NPA samples collected as part of our CAIR project in the period November 2006 to July 2015, no viruses or bacteria were detected in 649 samples (9%) and HBoV1 only was detected in 64 samples. This material was tested for all respiratory viruses mentioned in papers I-III, and in addition, an updated HRV PCR covering the recently discovered HRV-C-subtypes was included. Four-hundred and ninety two of the 649 samples (75.8%) with no viruses were from children with RTI, and 56 of the 64 samples (87.5%) with HBoV1 only, were from children with RTI ($p=0.03$, chi squared test) (unpublished data). This is an argument for specificity in the relationship between HBoV1 and RTI in children. A community-based study showing an even stronger association was recently published (233). It was conducted on people of all ages. A total of 3383 samples in this study were without any detected virus and among them 522 (15.4%) had RTI. One-hundred and fifty one samples were positive for HBoV1 and 69 (45.7%) of them had RTI ($p<0.001$, chi squared test). A weakness of this study was that multiple infections were not accounted for, meaning that the symptoms registered may have been caused by other viruses. Nevertheless, these data indicate that the frequency of RTI symptoms among HBoV1-positive people is higher than a presumptive basic level among people without any viruses detected with a broad and modern test panel.

5.8.4 Temporality

For this criterion to be fulfilled, the presence of HBoV1 should precede RTI. In other words, the cause should be present before the effect. Ideally, the virus should also be undetectable after the disease has resolved. As we have seen, HBoV1 can be present for months after an

acute infection, so we should not put much emphasis on this part of the criterion. Demonstration of appearance of a virus before symptoms arise, on the other hand, is important. It is an intuitive and central criterion in causality theory. A temporal relation between the appearance of HBoV1 and onset of RTI symptoms has been demonstrated. This was performed in follow-up studies based on both PCR and serology (152, 237). According to Martin et al's 2015-study, peak in fever registrations occurred three to five days after primary HBoV1 detection.

5.8.5 Biological gradient

If higher amounts of an agent are associated with stronger effects, meaning more severe disease, it may be used as an argument for causality. This is often called the dose-response effect, and it is central in pharmacological and toxicological causality research. In this context, it would mean an association between high viral load and more severe disease. As mentioned above, a peak in viral load usually occurs during the acute phase of a viral infectious disease. This is usually when the disease is at its most severe. Such dynamics lead to an association between viral load and disease severity when samples from different patients in different phases of the disease are compared. The dynamics are illustrated in figure 7. A correlation between viral load and disease severity has been demonstrated a few times for HBoV1. In our 2010 study (paper II), we showed that a high viral load ($>10^6$ copies/ml) was more common among children with LRTI than among children with URTI (OR 3.6, 95% CI: 1.2-10.7, p=0.02). By definition, the term LRTI only describes the localization of the infection, but LRTIs are usually more severe than URTIs and can be used as an indirect marker for disease severity. Four other hospital based studies have shown similar results. Three Chinese studies from 2013, 2014 and 2016, demonstrated associations between high viral loads ($>10^6$, $>10^7$ and $>10^6$ copies/ml, respectively) and higher severity scores (154, 317, 318). A Slovenian study from 2012 which included as many as 164 HBoV1-positive children showed that a high viral load ($>10^5$ copies/ml) was associated with a higher frequency of wheezing (p=0.037) (265). As already mentioned, a bias caused by more rapid hospital admittance for children with more severe disease is an alternative explanation for the differences. Hence, reproducibility of the findings ought to be tested in a community setting.

Another facet of the biological gradient argument is whether children who develop severe disease have a higher viral load at peak level than children who develop only mild disease. This would add weight to the argument, but follow-up studies designed to give information about this have not been published.

5.8.6 Plausibility and coherence

Plausibility and coherence are closely related criteria, and I will discuss them together. Both imply that the hypothesis about causality must fit with current biological knowledge. "Plausibility" means that the hypothesis must involve a plausible biological mechanism. The term "coherence" is more extensive, meaning that the causal explanation must fit with current theoretic models in biology.

The mechanism of disease is a well described one: A virus enters a host through the airway route, binds to specific target cells in one or more target organs, enters the cells and establishes symptomatic infections by eliciting cell and tissue damage in the target organs. This mechanism has been proven repeatedly over the past centrury for numerous viral infections. This has been proven also for closely related parvoviruses in both humans and other mammals. Consequently, the disease mechanism can be regarded as highly plausible.

Today, virological thinking is predominantly based on integrated virus-host models, applying evolutionary theory on both virus and host. According to the model, viral reproduction happens in the constant interplay with the host and its defense mechanisms. New mechanisms for increasing viral reproduction are constantly evolving, and the host organism simultaneously evolves, maximizing its own fitness and reproduction. The viral and host processes frequently stand in opposition to each other, and the result may be acute disease in the host at one end of the spectrum or silent elimination of the virus at the other. Alternatively, a delicate balance between the virus and host may develop. Models like these are the result of huge efforts in molecular biological research during the last few decades, revealing countless genetic mechanisms, signal pathways and biochemical processes. Different variants of the model are in use, depending on both virus and host. With parvoviruses in mind, I have made a simplified illustration of the dynamics of clinically important viral and host markers during an acute primary infection (Fig. 7). The figure is a synthesis of knowledge gathered over last few decades. Such data are available for a number of viruses and have been central in the development of current virus-host-interaction models. To fit with the model, the same temporal pattern of viral and host markers should be present during an HBoV1 infection.

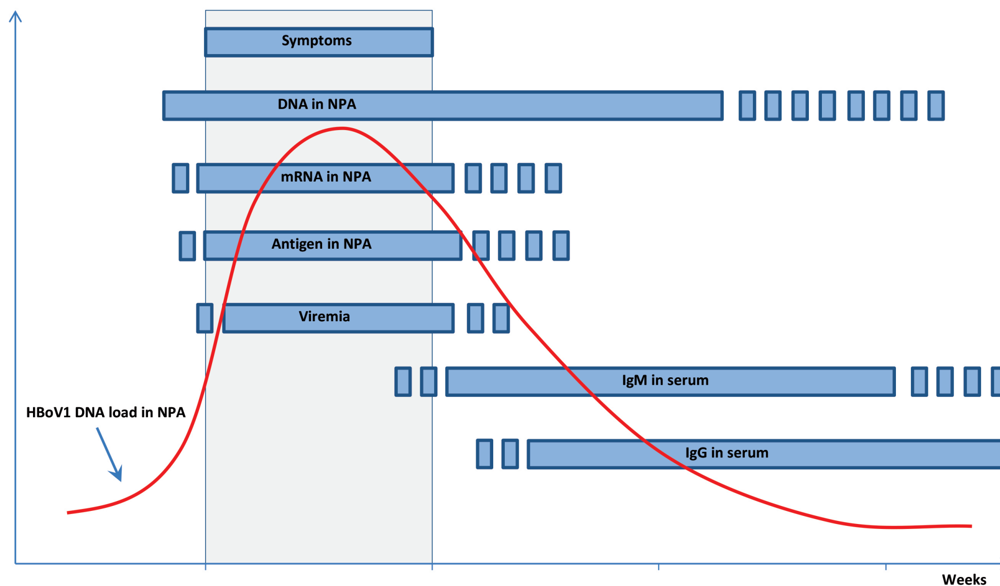

Figure 7. *T*emporal pattern of clinically relevant variables through the course of an acute primary HBoV1 infection. Development of HBoV1 DNA load in NPA is shown with the red line. All other parameters are represented qualitatively by blue bars. Dotted ends of the blue bars indicate variation or uncertainty.

Viral markers, such as HBoV1 DNA load, mRNA and viremia were described above. The evidence reviewed there shows that the markers behave according to the model. The HBoV1 antigen, on the other hand, has barely been studied. In a recently published case report, written in collaboration with Finnish and Dutch researchers, we describe HBoV1 antigen dynamics in a five-month-old girl admitted to hospital with LRTI (319). A newly developed HBoV1 antigen test was used to follow levels of the HBoV1-protein VP1 during the infectious process. Protein levels increased quickly after symptom start, reached a peak after three days, and had almost disappeared by day five. Samples had not been obtained following this. In addition, we have demonstrated good correlations between the presence of HBoV1 mRNA and HBoV1-VP1 in a material consisting of 55 respiratory samples, of which 23 were HBoV1 DNA-positive (320). Both results fit well with the model illustrated in figure 7.

The best markers of the host response are antibody or T-cell responses. This is because they are very virus specific, giving strong support to a link between the immune response and, specifically, the virus in question. Antibody responses are the easier of the two to measure and

are consequently the best studied. A number of studies have shown IgM- and IgG-responses in line with the model. In particular, a series of papers from Finland have given us important insights. Serological diagnosis of HBoV1 infection may be defined as either HBoV1-specific IgM-detection, seroconversion for HBoV1-specific IgG or at least a fourfold increase in IgG-titer (211). Given this definition, clear associations were found between serodiagnosis and HBoV1 viremia, monoinfection and a high HBoV1 load in NPA. These associations supported the idea that HBoV1 infection causes a specific immune response in the host. In addition, direct links between HBoV1-specific antibody responses and RTI in children have been demonstrated in two follow-up studies from the same research group (237, 243). They included samples from children in asymptomatic phases between RTIs, and demonstrated that RTI and AOM are associated with HBoV1 serodiagnosis . Kantola et al's 2015 study is worth special attention because they also accounted for cross-reactions between the four different HBoVs. By the use of absorption techniques they demonstrated that such cross-reactions occurred frequently, and probably have been a source of bias in most previous serology studies. This study, however, only demonstrated an association between HBoV1 serodiagnosis and AOM. This was probably due to low numbers of included patients giving low power to the study, and possibly due to recall bias. The study was primarily based on retrospective symptom reports from the parents, which were reported up to six months after the illness. Failure to remember all symptoms after such a period is likely. AOM, on the other hand, was diagnosed by physicians during the illness.

These results show accordance with the host part of the model illustrated in figure 7. By primarily describing host traits and not virus properties, the serology data represents a separate column to support the causality hypothesis.

Most viruses are very cell- and organ-specific. It is well known that specific interactions between viral surface proteins and proteins on target cells, so-called receptors, are important for viral entry into cells. Demonstration of viral presence in specific cells in the target organ and in none, or only few, other cells, would add support to the causality hypothesis. This would show coherence with current knowledge, and would, in addition, fit with a plausible mechanism of infection if viral presence is demonstrated in cells belonging to the respiratory tract in children, the expected route of entry for the viruses. However, histological examinations of respiratory tissue from infected children have not yet been published.

5.8.7 Experiment

At present, the experimental data are the weakest point in the argument for causality.

5.8.7.1 Cell and animal models

Very few experimental HBoV1 models have been established. No research animals have been found susceptible to the virus, and only laborious cell models based on primary human airway epithelial cells (HAE-cells) have been applicable. Standard commercial cell lines are not susceptible. A few studies on HBoV1 infection in HAE-cells have been published (206-209). They have predominantly been descriptive, showing cytopathogenic effects similar to the ones we see in airway epithelial cells in people with RTI. Effects reported include stretching of cells, cell hypertrophy, disruption of tight junctions between the epithelial cells and loss of cilia on the epithelial surface. These are all effects also seen with established airway pathogens, such as HRSV, in cell models, in animal models and in patients. All are known to be associated with the symptoms observed with RTIs. Although scarce, these data fit with the hypothesis that HBoV1 causes RTI in children. Moreover, the models will make future manipulation experiments such as loss of function studies on HBoV1 possible.

5.8.7.2 Human experiments

For obvious ethical reasons, challenge experiments on children cannot be performed. Intervention studies on children, on the other hand, are an alternative approach. If a vaccine or an antiviral agent reduces occurrence of, or morbidity with, HBoV1 infections, it would be a strong argument for the causality hypothesis. However, no vaccines or candidate antivirals are yet available.

5.8.8 Analogy

The analogy criterion implies that an equivalent system with similar pathogens should show similar clinical effects. In this context, it means that closely related parvoviruses should give similar symptoms in their respective hosts. The argument is weak, however, in a virological context. This is because viruses have very specific host relationships, varying considerably even among subtypes within the same species. Closely related subtypes of adenoviruses, for example, may have very different host ranges. In addition, they may cause very different symptoms in the same host. It is nevertheless worth noting that two bocaviruses closely related to HBoV1, bovine parvovirus and canine minute virus, cause gastrointestinal and respiratory diseases in cattle and dogs, respectively (321, 322). We have too little knowledge

about the pathogenic potential of HBoV1's closest relatives HBoV2-4 and gorilla bocavirus to use them in analogy arguments.

5.8.9 Conclusion

The epidemiological data from thoroughly controlled studies or follow-up studies all point in the same direction: HBoV1 causes RTI in children very early in life, usually during the first two years. The total body of evidence gives strong support to the hypothesis that there is a causal relation between HBoV1 and RTI in children. Furthermore, the data tells us that HBoV1 DNA is common in children with RTIs caused by other viruses and also in healthy children. This makes diagnosis of HBoV1 infections challenging. It is clear that the diagnostic value of qualitative HBoV1 DNA detection in NPA by PCR is low. A better approach is needed. Quantitative DNA detection in NPA with a cut-off, HBoV1 mRNA detection in NPA or HBoV1 DNA detection in plasma are all good candidates. Further studies on sensitivity and specificity are needed before we can recommend a strategy for clinical use. My current suggestion is to use a quantitative HBoV1 DNA PCR on NPA with a cut-off of 10^6 copies/ml. Before the clinical sensitivity of the HBoV1 mRNA test or HBoV1 viremia is determined, this will be the most flexible approach giving the clinician the opportunity to evaluate results close to cut off together with clinical data.

HBoV1 should now be included among the RTI-causing viruses and in standard test panels for RTI in children. Indications for testing, however, are still a matter for discussion. At present, I would suggest only testing for HBoV1 in hospitalized children with LRTI. When specific treatment or prophylaxis options are lacking, testing in a community setting will have few consequences.

5.9 Future prospects

In medical research on viruses, it is a necessary first step to determine their pathogenic potential. Interesting basic biological principles may be discovered on the way, but the main focus has to be characterization of human disease, and search for treatment options. The next steps along this line of thought for HBoV1 will be to further characterize diseases caused by the virus, and to search for the best diagnostic tools for use in clinical practice. Basic research on cell models for HBoV1 are in the starting pits and significant advances in our knowledge about the basic biology of the virus can be expected soon. Aside from widening our horizon on biology, basic research is a necessary prerequisite for finding targets for treatment or prophylaxis.

Today, the CAIR material consists of samples from about 4000 patients and 700 controls included with informed consent. This material will enable us to characterize clinical manifestations of HBoV1 infections in detail. Studies on this are under way. Based on the same data, we are also planning a study on diagnostic performance of several HBoV1-related markers.

Additionally, we have commenced a project on full genome sequencing of HBoV1 strains from the CAIR material. Eighteen full-genome sequences have been obtained, and mutation analyses are in progress. These analyses will enable us to conduct detailed molecular epidemiological characterizations, and to search for virulence factors. A starting point will be to look for mutations in the genomes associated with disease per se or with severity of disease. This may become a major focus of our research in the future. The CAIR material gives us invaluable opportunities for linking sequence data with clinical information.

Moreover, the CAIR group is now initiating a long term follow-up study on children with asthma predispositions. The main focus of the project will be associations between HRSV-, HRV- and HMPV infections and asthma development in children. At a later stage, the material will enable us to study the role of other viruses, including HBoV1, in the process.

HBoV1 has proven to be a good model virus for studying the complex world of respiratory viruses in children. HBoV1 research has been central in revealing that every child harbors a mixture of viruses from time to time, even when they are symptomless. Lessons learned from studies on HBoV1 may have implications for research on other viruses behaving similarly, such as HRVs, HAdVs, coronaviruses and enteroviruses.

6 References

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Paper I

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Human bocavirus commonly involved in multiple viral airway infections

Andreas Christensen[a,*], Svein Arne Nordbø[a,c], Sidsel Krokstad[a], Anne Gro Wesenberg Rognlien[b], Henrik Døllner[b,c]

[a] *Department of Medical Microbiology, St. Olavs Hospital, Trondheim University Hospital, Norway*
[b] *Department of Pediatrics, St. Olavs Hospital, Trondheim University Hospital, Norway*
[c] *Institute of Laboratory Medicine, Children's and Women's Health, Norwegian University of Science and Technology, Norway*

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Abstract

Background: Human bocavirus (HBoV) was recently discovered in children with acute respiratory tract infections. We have included a PCR for HBoV in a study on airway infections in children.
Objectives: To study the occurrence of HBoV in Norwegian children, and to evaluate the results of a semiquantitive PCR.
Study design: During a 4-month period in the winter season 2006/2007 we collected nasopharyngeal aspirations from children who were admitted to the Department of Pediatrics. All samples were examined for 17 agents with real-time PCR.
Results: HBoV was detected in 45 of 376 samples (12%). The occurrence of HBoV was stable during the study period. Multiple viral infections were present in 78% of the samples (42% double, 20% triple and 16% quadruple infections). RS-virus, enterovirus and human metapneumovirus were the most frequently codetected agents. In samples with a high load for HBoV, significantly fewer multiple infections were found than in the other samples. Eighty-eight percent of the 25 patients with HBoV recorded as either the only or the dominating virus, and 50% of the other patients, had lower respiratory tract infection. The difference was statistically significant.
Conclusions: HBoV was frequently detected in nasopharyngeal aspirates from children with airway infections in Norway. Multiple viral infections were common among the HBoV-infected patients. Semiquantitive PCR results may be useful for interpretation of clinical relevance.
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Keywords: Human bocavirus; Airway; Infection; Multiple; Children

1. Introduction

Human bocavirus (HBoV) was discovered in 2005 (Allander et al., 2005). It belongs to the family *parvoviridae,* and it is the second virus in this family to be associated with human disease (after parvovirus B19). Little is known about the virus' kinetics and cell tropism.

HBoV is common in airway samples from children less than 5 years with respiratory tract infections, and it has a worldwide distribution (Allander et al., 2007; Arnold et al., 2006; Arden et al., 2006; Fry et al., 2007; Manning et al., 2006; Regamey et al., 2007). A causal relationship between HBoV and airway infection has not been established yet, but some controlled studies supporting this hypothesis have been published (Allander et al., 2007; Fry et al., 2007; Kesebir et al., 2006; Maggi et al., 2007; Manning et al., 2006).

We have studied the occurrence of HBoV in Norwegian children with respiratory tract infections, and evaluated the semiquantitative PCR-results against clinical manifestations.

2. Methods

We collected nasopharyngeal aspirates from children who were admitted to the Department of Pediatrics, St. Olavs Hospital, Trondheim University Hospital with respiratory tract infections during the time period November 13, 2006 to March 16, 2007. St. Olavs Hospital is the regional hospital for Mid-Norway covering a population of 640 000.

* Corresponding author. Tel.: +47 90554486; fax: +47 72576417.
E-mail address: andreas.christensen@stolav.no (A. Christensen).

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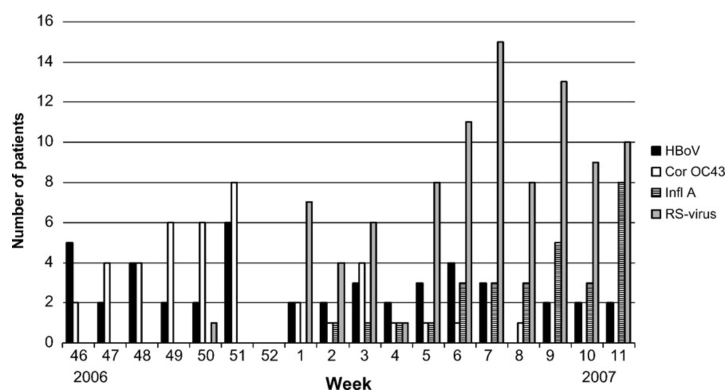

Fig. 1. Number of human bocavirus (HBoV), coronavirus OC43 (Cor OC43), influenza A-virus (Infl A) and RS-virus per week.

Clinical data were obtained from medical records. The children were classified in two main diagnosis categories: lower respiratory tract infection (LRTI) and upper respiratory tract infection (URTI). Other diagnoses not mentioned in the statistics included tonsillitis, gastroenteritis and fever. LRTI was diagnosed in the presence of dyspnea, signs of lower airway obstruction (wheezing, retractions) and/or a positive radiogram (infiltrates, atelectasis, air trapping). URTI was diagnosed when rhinitis, pharyngitis and/or otitis media was present in the absence of signs of LRTI.

Using PCR the nasopharyngeal aspirations were examined for adenovirus, HBoV, coronavirus (OC43, 229E and NL63), enterovirus, human metapneumovirus, influenza A and B virus, parainfluenza virus type 1-3, RS-virus, rhinovirus, *Bordetella pertussis*, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*. All PCRs were real-time assays based on TaqMan probes. The target for the HBoV-PCR was the NP-1-gene. Selections of primers and probe were based the sequences published by Allander et al. (2005). (Forward primer: CCA CGT GAC GAA GAT GAG CTC, reverse primer TAG GTG GCT GAT TGG GTG TTC, probe CCG AGC CTC TCT CCC CAC TGT GTC G, 5′6-FAM, 3′ TAMRA.) The amount of virus in each sample was recorded semiquantitatively based on the C_t-value (cycle threshold value) and grouped in three categories (high, medium and low viral load). The break points were set to C_t 28 and C_t 35. HBoV was recorded as the dominating virus in a sample when the C_t-value was at least three cycles lower than the C_t-value for any other virus.

In addition all samples were collected on ordinary virus transport media without antibiotics and cultured for viruses and bacteria with standard methods.

3. Results

HBoV was detected in 45 of 376 nasopharyngeal aspirates (12%). It was the fourth most common virus in the material after RS-virus (25%), rhinovirus (17%) and human metapneumovirus (14%). Other common viruses in the material were enterovirus (11%) and coronavirus OC43 (11%). During the 4-month study period the occurrence of HBoV was stable, in contrast to coronavirus OC43, influenza A and RS-virus, which varied significantly (Fig. 1).

At least one virus was detected in 78% of the 376 samples in the total material. One solitary virus was found in 50%,

Table 1
Number of codetected viruses in 35 HBoV-infected patients who had multiple viral infections

	Number of detections			Part of triple/quadruple infections
	Total	PCR	Culture	
Adenovirus	6	5	3	4
Coronavirus OC43	10	10	–	6
Coronavirus NL63	1	1	–	–
Enterovirus	9	9	1	8
Human metapneumovirus	7	7	1	4
Influenza virus A	3	3	3	3
Parainfluenzavirus type 3	5	5	2	3
Rhinovirus	10	10	–	8
RS-virus	8	8	7	3
Cytomegalovirus	2	ND[a]	2	1

The number of detections made by PCR and viral culture are shown. Right column: number of samples with at least two viruses detected in addition to HBoV.
[a] Not done.

Table 2
The eight most common viruses detected in 376 nasopharyngeal samples categorized after number of viruses detected per sample

	Total ($n = 376$)		Double infection ($n = 77$)		Triple infection ($n = 19$)		Quadruple infection ($n = 7$)	
	n	(%)	n	(%)	n	(%)	n	(%)
Adenovirus	24	6	9	12	6	32	3	43
Coronavirus OC43	41	11	18	23	6	32	4	57
Enterovirus	42	11	16	21	10	53	5	71
HBoV	45	12	19	25	9	47	7	100
HMPV	54	14	20	26	6	32	2	29
Influenza A-virus	29	8	7	9	3	16	1	14
Rhinovirus	65	17	19	25	8	42	4	57
RSV	93	25	29	38	6	32	1	14

Table 3
The HBoV-positive samples ($n = 45$) categorized after viral load and whether codetected viruses were present or not

HBoV-load	HBoV only	Multiple infection	Total
High	9	13	22
Medium/low	1	22	23

two viruses in 21%, three viruses in 5% and four viruses in 2% of the samples.

Coinfections were detected in 78% of the 45 HBoV-positive samples. 42% of the samples contained one other virus, 20% contained two and 16% contained three other viruses. Table 1 shows the distribution of the codetected viruses. HBoV was more frequently detected in samples from patients with multiple infections. The proportion of samples containing HBoV increased with the number of viruses found per sample (Table 2).

Based on semi quantitative evaluation HBoV was found to be the dominating virus in 15 of the 35 patients with multiple viral infections (43%). In the samples with a high load for HBoV significantly fewer multiple infections were found ($p < 0.001$, Fisher's exact test) (Table 3). The same tendency was found for RS-virus, although not significant.

Seventy-one percent of the HBoV-infected children had LRTI (bronchiolitis: 44% and pneumonia: 27%) and 20% had URTI. For the patients with HBoV recorded as either the dominating or the only virus the proportion of LRTI was 88%. In the group where HBoV was not the dominating agent the proportion was 50% (Table 4). The difference was statistically significant ($p = 0.005$, Fisher's exact test).

The median age of the patients with HBoV-infection was 17 months. 58% were males.

Table 5 shows the results of the bacterial cultures. The bacteria were equally distributed in the diagnosis groups, and no association was found between viral and bacterial agents. For the majority of the patients who probably had a bacterial pneumonia, the bacterial culture was negative. These patients had received antibiotics before admission.

Table 4
The HBoV-positive samples ($n = 45$) categorized after diagnosis group and whether HBoV was the dominating agent or not

	LRTI	URTI	Other	Total
HBoV only/dominating virus	22 (88)[a]	1(4)	2(8)	25
HBoV not dominating	10 (50)	8(40)	2(10)	20

[a] Numbers in parentheses, percent.

4. Discussion

We found HBoV in 12% of the samples, which is a high number compared to other hospital based studies on respiratory infections in children (Arden et al., 2006; Arnold et al., 2006; Kesebir et al., 2006).

No evidence for a seasonal appearance of HBoV was found in our material, but the study period was too short for any conclusions to be made. However, the period was long enough to show a clear seasonality for coronavirus OC43, RS-virus and influenza A-virus.

With newer highly sensitive PCR-based methods, the number of viral detections in airway samples has increased considerably. A striking finding is the high number of coinfections. This is a common finding in HBoV-infected patients (Allander et al., 2007; Manning et al., 2006). In our study 78% of the HBoV-infected children had multiple viral infections. Coronavirus OC43, rhinovirus, enterovirus and RS-virus were the most frequently codetected viruses. We could not find any association between detection of HBoV and other specific viruses. The proportion of multiple viral infections in HBoV-infected patients has varied between 35 and 83% in other studies (Allander et al., 2007; Choi et al., 2006; Foulongne et al., 2006; Fry et al., 2007).

HBoV-infections were more frequent when the number of coinfections was high. In the total material ($n = 376$) HBoV was detected in all seven samples containing four viruses, and in half of the samples containing three viruses. Similar figures were found for enterovirus, rhinovirus and coronavirus OC43. In contrast, this tendency was not demonstrable

Table 5
Results of bacterial cultures from the 45 samples containing HBoV

	n	(%)
S. pneumoniae	13	29
H. influenzae	8	18
M. catharralis	4	9
Normal flora	4	9
Negative culture	16	36

for RS-virus and influenza A-virus. Long-time shedding of HBoV, enterovirus, coronavirus OC43 and rhinovirus is a possible explanation. When a single sample can contain as many as four or more viruses it becomes difficult to evaluate the clinical significance of each virus. One way to solve this problem may be to quantify the individual viral nucleic acids. Quantitative analysis of nasopharyngeal aspirates is methodologically difficult because the quality of the specimens obtained varies from person to person depending on sampling technique and the condition of the patients' nasal mucosa. An exact quantitation may consequently give misleading results. We have therefore chosen to do a semiquantitive analysis and roughly group the results in three categories. Our results indicate that this approach can be fruitful. In samples with a high load of HBoV few other viruses were detected. This indicates that assessment of viral load may be of clinical relevance, but a prospective study including clinical data and a control group is needed for this question to be fully addressed.

Most of the HBoV-infected children had LRTI. This is in accordance with other studies (Arnold et al., 2006; Manning et al., 2006). The proportion of pneumonias was relatively high (27%). This may be a coincidental finding as more than half of the pneumonia patients probably had bacterial pneumonia. We found a higher proportion of LRTI in the patients where HBoV was the only or the dominating virus, indicating a tendency to cause lower respiratory tract infections.

The median age of 17 months is higher than the typical median age for RS-virus and human metapneumovirus (Døllner et al., 2004). Whether this is a result of differences in epidemiology or in virus kinetics remains to be elucidated. It is not known for how long time the virus can be detected in airway specimens. Allander et al. (2007) found HBoV in 19% of blood samples three weeks after an acute infection indicating that viremia can persist for some time after an acute infection.

Serum, urine and feces were also collected from some of the HBoV-positive patients. This was not done systematically, and many patients were discharged before the samples could be obtained. We detected HBoV in serum from four patients, all of whom had a high viral load. HBoV was detected in urine from two of these patients. This indicates that HBoV gives a viremia during acute infection and that shedding through urine can happen. HBoV was found in feces from two patients. One of them had gastroenteritis. In the fecal sample from this patient rotavirus was also detected giving a probable explanation for the gastroenteritis. In a recent study HBoV was proposed as a cause of gastroenteritis (Vicente et al., 2007). We therefore examined 101 fecal samples from unselected patients of all age groups with gastroenteritis. Only one specimen was positive for HBoV. This was a 1-year-old boy who also tested positive for rotavirus. Furthermore, he had a respiratory illness four weeks before the gastroenteritis. Thus, our data do not support the hypothesis that HBoV can cause gastroenteritis.

Our results indicate that semiquantitative recording of HBoV-PCR-results have clinical relevance. We have recently started a prospective study including a control group to explore this issue further.

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Paper II

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Human bocavirus in children: Mono-detection, high viral load and viraemia are associated with respiratory tract infection

Andreas Christensen[a,c,*], Svein Arne Nordbø[a,c], Sidsel Krokstad[a], Anne Gro Wesenberg Rognlien[b], Henrik Døllner[b,c]

[a] Department of Medical Microbiology, St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway
[b] Department of Pediatrics, St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway
[c] Institute of Laboratory Medicine, Children's and Women's Health, Norwegian University of Science and Technology, Trondheim, Norway

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ABSTRACT

Background and objectives: Human bocavirus 1 (HBoV1) has recently been detected in children with respiratory tract infections (RTI). In order to study whether HBoV1 can cause RTI, we investigated its presence in children with upper RTI (URTI), lower RTI (LRTI) and a control group of children without RTI.
Study design: Nasopharyngeal aspirates (NPA) and blood samples were collected from children admitted to hospital with RTI from 6 June 2007 to 28 February 2009 ($n = 1154$), and from children admitted for elective surgery who had no RTI ($n = 162$). Using polymerase chain reaction (PCR), the NPAs were examined for 17 infectious agents including HBoV1. Blood samples were tested with HBoV1-PCR only.
Results: HBoV1 was detected in NPAs from 10% of patients and 17% of controls. Adjusted for age, gender and the presence of other viruses, HBoV1 was not associated with RTI. In the HBoV1-positive NPAs, at least one other virus was detected in 75% and the virus appeared alone in 25%. Adjusted for age and gender, the detection of HBoV1 as the sole virus was associated with RTI, but not with LRTI. Viraemia was found only in children with RTI. The study showed that it was associated with RTI and LRTI. A high HBoV1-load was associated with LRTI, but not with RTI. No interactions between HBoV1 and other infectious agents were found.
Conclusions: Our data support the hypothesis that HBoV1 causes RTI in children, because detection of HBoV1 alone, viraemia and high viral load are associated with RTI and/or LRTI in this age group. However, HBoV1 is common in healthy children.

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1. Background and objectives

The human bocavirus, belonging to the family *Parvoviridae* and the genus bocavirus, was discovered in 2005.[1] Four different species of human bocavirus have been proposed, and the name human bocavirus 1 (HBoV1) has now been suggested for the originally discovered virus.[2] It has been associated with respiratory tract infections (RTIs) in children and a link to more complex clinical conditions in immunosuppressed patients has been suggested.[3–5] However, HBoV1 is frequently found together with other respiratory viruses and consequently its causative role in childhood RTI may be questioned. Possible interactions between HBoV1 and other viruses have also been discussed.[6] We studied the role of HBoV1 in childhood RTI by comparing its presence in children hospitalised with RTI with a control group of symptom-free children admitted to elective surgery. We also looked for several other respiratory pathogens in order to evaluate their coexistence with HBoV1.

2. Study design

The study was performed at the Department of Paediatrics, St. Olavs Hospital, Trondheim University Hospital during the period 6 June 2007 to 31 August 2009. St. Olav's Hospital is a regional hospital for mid-Norway covering a population of 640 000. As part of routine clinical work at our department, and on the discretion of the medical doctors, nasopharyngeal aspirates (NPAs) were collected from most children who were admitted with RTI. The parents were informed about the study and asked to participate. In addition to the NPA, a blood sample for the study was collected simultaneously with routine blood samples from included children.

The children were classified as having either lower or upper respiratory tract infection (LRTI; URTI). LRTI was diagnosed in the presence of dyspnoea, signs of lower airway obstruction (wheezing, retractions) and/or a positive chest X-ray (infiltrates, atelectasis and

* Corresponding author at: Department of Medical Microbiology, St. Olavs Hospital, Trondheim University Hospital, Trondheim, Norway. Tel.: +47 90554486; fax: +47 72576417.
E-mail address: andreas.christensen@stolav.no (A. Christensen).

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air trapping). URTI was diagnosed when rhinitis, pharyngitis and/or otitis media were found without signs of LRTI.

A control group (n = 162) was included prospectively in the same time period. The controls were children admitted for elective surgery who had no symptoms of RTI in the last 2 weeks. Every week throughout the study period we asked the parents of 2–4 children to participate. Most controls had surgery for cryptorchidism, hernia repair or benign skin tumours, and none for ear, nose and throat surgery. A total of 1316 samples were included in the study, 1154 from patients and 162 from controls. The mean ages in the patient and control groups were 35 and 43 months (p = 0.05).

Using polymerase chain reaction (PCR), the NPAs were tested for adenovirus, HBoV1, coronavirus (OC43, 229E and NL63), enterovirus, human metapneumovirus, influenza A and B virus, parainfluenza virus type 1–3, RS-virus (RSV), rhinovirus, *Bordetella pertussis*, *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*. Nucleic acid was extracted with NucliSens®easyMag® (bioMerieux). All PCRs were in-house real-time assays based on TaqMan probes. The analyses were carried out as part of the daily laboratory routine and mainly performed within 24 h after sample collection. The target for the HBoV1-PCR was the NP-1 gene; the primers and probe have been previously described.[7] Quantitative standards for the real-time HBoV1-PCR assay were made by cloning a plasmid (pCR®4-TOPO®) containing the PCR product. The amount of nucleic acid was measured and serial dilutions covering a range of seven logs were made. The viral load in each sample was recorded semi-quantitatively and grouped in three categories: high viral load (10^6–10^{10} copies ml^{-1}), medium viral load (10^4–10^6 copies ml^{-1}) and low viral load (10^3–10^4 copies ml^{-1}). The reportable range of the assay was from 1000 copies ml^{-1} (20 copies per reaction) to 10^{10} copies ml^{-1}. In addition, plasma samples available from 60 of the 144 HBoV1-positive children were examined with the HBoV1-PCR.

All NPAs were collected in ordinary virus transport media without antibiotics and were cultured for viruses using standard cell lines. The transport media were also used to culture bacteria using standard agarose media. Growth of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catharralis* were recorded.

Plasma from a patient in whom HBoV1 had been detected in blood was diluted 1:1000 and pre-centrifuged at 16 000 × g for 30 min. 175 μl of the supernatant was then ultra-centrifuged at 27 psi (90 000 rpm) directly to 400 mesh carbonated formvar-covered copper grids and stained with 2% phosphotungstic acid. The grids were examined at magnifications 50 000× to 200 000× with Jeol JEM-1011 (Jeol Ltd.).

Statistical analysis was done by Chi-squared test for categorical data and Student's t-test for continuous data. Multiple logistic regression analysis was used to evaluate the association between HBoV1 and RTI, controlling for differences in age, gender and the presence of other viruses among cases and controls. We report the odds ratio (OR) with 95% confidence interval (95% CI) and the corresponding p-value as a measure of the strength of the association. All analyses were performed using SPSS software version 15 (Statistical Package of Social Science Inc.).

3. Results

In all, 144 of 1316 samples (11%) were positive for HBoV1. Fewer HBoV1-positive samples were found in the summer months, but the difference was not significant when adjusted for the number of samples received each month (Fig. 1). In total, 63% of the children were boys (61% in the patient group and 75% in the control group). The mean age of the HBoV1-positive patients was 19 months (SD: 10.6 months) and of the controls, 33 months (SD: 14.2 months) (p < 0.001). One-hundred and seventeen of 1154 samples (10%) from children with RTI were positive for HBoV1. Of these, 40 (37%) had URTI and 68 (63%) LRTI (of which 49% had bronchiolitis and 14% pneumonia). Of the 162 controls, 27 (17%) were positive for HBoV1. In a multiple logistic regression analysis adjusting for age, gender and the presence of other respiratory viruses, we found no association between a positive HBoV1-PCR test in NPA and RTI (OR: 0.8, 95% CI: 0.5–1.3, p = 0.30).

Among the 144 HBoV1-positive NPA samples, HBoV1 was detected alone in 25% (36 of 144). Twenty-nine percent (34 of 117) of the patient samples were positive for HBoV1 alone and 7% (2 of 27) of the controls (p = 0.02). One additional virus was found in 46%, two in 18%, three in 10% and four in 1% (n = 144). Rhinovirus, enterovirus, adenovirus and RSV were the most commonly co-detected viruses (Table 1). A logistic regression analysis on the HBoV1-positive samples, adjusted for age and gender, showed that detection of HBoV1 alone was associated with RTI (OR: 5.2, 95% CI: 1.1–24.4, p = 0.04) but not LRTI (OR: 0.6, 95% CI: 0.2–1.4 p = 0.20).

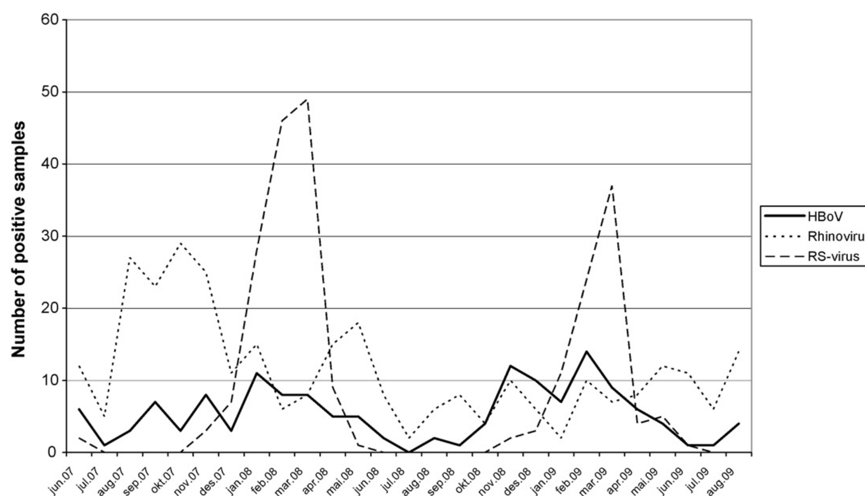

Fig. 1. Monthly distribution of samples positive for HBoV1, rhinovirus and RSV, in the period June 2007–August 2009.

Table 1
Viruses codetected in HBoV1-positive samples from patients with respiratory tract infection (RTI) ($n = 117$) and controls ($n = 27$).

Virus	Total		RTI patients		Controls	
	n	(%)	*n*	(%)	*n*	(%)
Adenovirus	30	(20.8)	21	(17.9)	9	(33.3)
Coronavirus 229E	1	(0.7)	1	(0.9)	0	(0)
Coronavirus OC43	7	(4.9)	6	(5.1)	1	(3.7)
Coronavirus NL63	2	(1.4)	1	(0.9)	1	(3.7)
Enterovirus	39	(27.1)	25	(21.4)	14	(51.9)*
Influenzavirus A	1	(0.7)	1	(0.9)	0	(0)
Influenzavirus B	1	(0.7)	1	(0.9)	0	(0)
Metapneumovirus	5	(3.5)	5	(4.3)	0	(0)
Parainfluenzavirus type 1	1	(0.7)	1	(0.9)	0	(0)
Parainfluenzavirus type 2	0	(0)	0	(0)	0	(0)
Parainfluenzavirus type 3	6	(4.2)	6	(5.1)	0	(0)
Rhinovirus	42	(29.2)	32	(27.4)	10	(37.0)
RS-virus	23	(16.0)	23	(19.7)	0	(0)*

* $p < 0.05$.

A high viral load (>10^6 copies ml^{-1}) in the NPA was found in 33% (39 of 117) of patients and 15% (4 of 27) of controls. However, when we adjusted for age, gender and presence of other viruses, a high viral load was not associated with RTI (OR: 1.4, 95% CI: 0.4–5.1, $p = 0.57$). A very high viral load (>2×10^8 copies ml^{-1}), though, was clearly associated with RTI, as no controls and 14 patient samples had a copy number higher than this. LRTI was found in 82% (28 of 34) of the patients with a high viral load (>10^6 copies ml^{-1}) and in 54% (40 of 74) of patients with moderate or low viral load. (Nine children with complex clinical conditions were excluded from this analysis.) Adjusted for age, gender and other viruses, a high viral load was associated with LRTI (OR: 3.6, 95% CI: 1.2–10.7, $p = 0.02$).

HBoV1-viraemia was found in 45% of patients with available samples (18 of 40) and in none of the controls (0 of 20). Viraemia was almost exclusively detected in patients younger than 2 years (16 of 18). More children with LRTI (57%, 16 of 28) than URTI (20%, 2 of 10) had viraemia ($p = 0.04$). (Two children with complex clinical conditions were excluded from this analysis.) Furthermore, viraemia was present more frequently in patients with a high viral load in NPA (70%, 14 of 20) compared to patients with a moderate or low viral load (10%, 4 of 40) ($p < 0.001$). Thirty-three percent (6 of 18) of the patients with viraemia had HBoV1 alone in the NPA, compared to 10% (4 of 42) of the patients without viraemia ($p = 0.052$, Fisher's exact test). Rhinovirus (33%, 6 of 18) was the most commonly co-detected virus among the viraemic patients.

Electron microscopy examination of blood from a boy of 18 months with bronchiolitis, HBoV1 alone and a high HBoV1-load in NPA and blood, showed viral particles with size ~25 nm, compatible with human bocavirus (Fig. 2).

Adenovirus and enterovirus were more frequent in the HBoV1-positive NPA samples than the negative ones (Table 2). In contrast, two other commonly detected viruses, rhinovirus and RSV, were as common in the HBoV1-positive as the negative ones. The frequency of RTI among children positive for rhinovirus, RSV, enterovirus or adenovirus was not affected by the simultaneous presence of HBoV1.

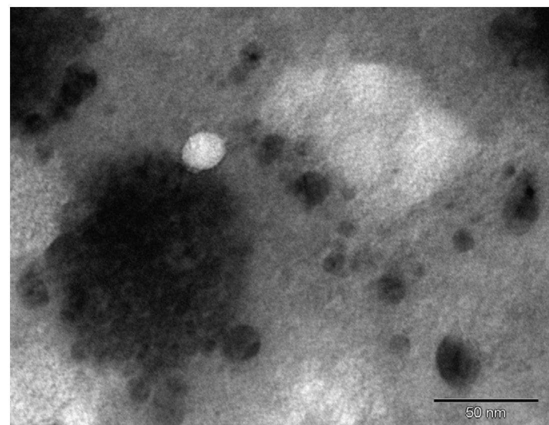

Fig. 2. Electron microscopic image taken of serum from a boy of 18 months with HBoV1-infection showing a viral particle with size ca. 25 nm. The image is taken at 200 000× magnification.

When bacteria were considered in addition to viruses, multiple detection was made in 85% of the HBoV1-positive NPA samples. One patient had *M. pneumoniae*. *S. pneumoniae*, *H. influenzae* and *M. catharralis* were present in 26%, 19% and 27% of the HBoV1-positive samples, respectively. There were no differences in the distribution

Table 2
The four viruses most commonly co-detected with HBoV1 distributed by study group and whether HBoV1 was present ($n = 144$) or not ($n = 1172$).

		Total population ($N = 1316$) *n* (%)[a]		Patient group ($N = 1154$) *n* (%)[b]	Control group ($N = 162$) *n* (%)[b]	
Adenovirus	HBoV1 present	30 (20.8)	$p < 0.001$	21 (70.0)	9 (30.0)	$p = 0.19$
	HBoV1 not present	91 (7.8)		74 (81.3)	17 (18.7)	
Enterovirus	HBoV1 present	39 (27.1)	$p < 0.001$	25 (64.1)	14 (35.9)	$p = 0.53$
	HBoV1 not present	111 (9.5)		78 (70.3)	33 (29.7)	
Rhinovirus	HBoV1 present	42 (29.2)	NS	32 (76.2)	10 (23.8)	$p = 0.17$
	HBoV1 not present	266 (22.7)		225 (84.6)	41 (15.4)	
RSV	HBoV1 present	23 (16.0)	NS	23 (100.0)	0 (0.0)	$p = 0.41$
	HBoV1 not present	207 (17.7)		201 (97.1)	6 (2.9)	

[a] Percentage of corresponding virus within the two groups either positive ($n = 144$) or negative ($n = 1172$) for HBoV1.
[b] Percentage within row showing the distribution of the co-detected virus between patient group and control group.

of bacteria among the HBoV1-positive and negative samples, or between patients and controls (data not shown).

4. Discussion

Our study confirms that HBoV1 is frequently found in children with RTI, and often simultaneously with other respiratory viruses. In contrast to most other studies, we also detected HBoV1 in many children without RTI.[3,8–10] Nevertheless, our findings indicate that HBoV1 causes disease, because detection of the virus alone, a high viral load in NPAs and viraemia were associated with RTI in hospitalised children.

HBoV1 was detected alone in a third of the patients but only in a few of the controls, and even if the majority in both groups had multiple viruses, this finding supports a causal role of HBoV1 in relation to RTI. HBoV1 alone was not associated with a higher occurrence of LRTI. This finding has been reported previously.[9,11] We did not control for duration of symptoms before admission, and this may be an explanation because duration of symptoms and disease severity are likely to be related.

We were surprised to find no association between a high viral load ($>10^6$ copies ml^{-1}) in the NPAs and RTI. This may also be due to the fact that we did not control for duration of symptoms before admission. However, an even higher viral load ($>2 \times 10^8$ copies ml^{-1}) was seen only in children with RTI. Furthermore, an association was found between a high viral load ($>10^6$ copies ml^{-1}) and infection in the lower respiratory tract. This finding may indicate that high viral load is associated with more severe disease, and may represent a dose-effect argument for a causal relation between HBoV1 and RTI.

The detection of HBoV1-DNA in plasma may represent viraemia or simply leakage of DNA from infected cells. Fig. 2 shows the presence of viral particles in blood from one patient. This may suggest that detection of HBoV1-DNA in blood represents a true viraemia, but this singular finding should be studied on a larger scale. Nevertheless, as it has been shown before,[9,12] HBoV1-viraemia was strongly associated with RTI in general and, although weaker, with LRTI. It is most likely that a viraemia with HBoV1 indicates a present HBoV1-infection.

Another characteristic feature of HBoV1-viraemia in our study was that it was almost exclusively seen in children of less than 2 years, and thus may be a marker for primary HBoV1-infection. Serological studies show a correlation between IgM-response, IgG-seroconversion and an episode of HBoV1-viraemia related to RTI in small children.[11,12] At the age of three, 70–90% of children are seropositive for HBoV1,[11,13] suggesting that the infection in early childhood gives immunity against later infections. Most patients with viraemia, high viral load and LRTI in our study were in the 12–17 months age group, the age at which most children have lost the protection of maternal antibodies. In the older children, however, the majority of the HBoV1-detections could represent asymptomatic re-infections, reactivations of latent/persistent infections or long-time virus shedding. Reactivation of other human pathogenic parvoviruses is known to happen.[14,15] Further studies on this issue are needed.

Most previous studies detected HBoV1 among only a few controls,[3,8–10] but two reported similarly high rates to ours of HBoV1-positive samples in healthy children.[16,17] The major strengths of our study are the large case group and the prospective inclusion of controls from the same time period and same geographical area, and the use of the same sampling technique and test algorithm for both groups. Patients admitted for ear, nose and throat surgery were not included in the control group because many of these diseases may be related to viral infections. The main differences between cases and controls that might influence the evaluation of HBoV1 were age, gender and the presence of other viruses. In the analyses, we used multiple logistic regression analysis to adjust for these factors.

We detected multiple viruses in three quarters of the nasopharyngeal samples, as have other PCR-based studies.[9,18] Adenovirus and enterovirus were more common in children with HBoV1. Enterovirus can be detected in samples from patients several weeks after infection,[19] and shedding of HBoV1 over long periods has recently been documented.[17,20] Thus, HBoV1 and enterovirus seem to share a common tendency for prolonged shedding, which may explain their frequent co-detection. A criterion of 2 weeks without respiratory symptoms before inclusion in the control group is, in this context, relatively short. Long-time virus shedding is therefore a plausible explanation for the high occurrence of HBoV1 in our control group. It has previously been shown that strong immune stimuli can cause reactivation of adenovirus in adenoid tissue,[21] and therefore we speculate that the association between HBoV1 and adenovirus may be associated with reactivation of the latter. Reactivation of HBoV1, an alternative or supplementary explanation, has yet to be demonstrated. As expected, RSV was common in children with RTI and rare among controls, appearing at similar rates in children with or without HBoV1, which indicates the dominance of RSV. Similarly, for each of the other viruses studied, co-detection of HBoV1 did not increase the frequency of RTI (Table 2). Cultures for bacteria were included in the study in order to look for interactions with viruses, but no such interactions were seen for HBoV1. Therefore, our findings do not support the existence of significant interactions between HBoV1 and other respiratory viruses or bacteria. However, more studies are needed to clarify these complex matters.

Conflict of interest

None.

Funding

None.

Ethical approval

Approved by the Regional Committee for Medical and Health Research Ethics in Mid-Norway.

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Paper III

Detection of Spliced mRNA from Human Bocavirus 1 in Clinical Samples from Children with Respiratory Tract Infections

Andreas Christensen, Henrik Døllner, Lars Høsøien Skanke, Sidsel Krokstad, Nina Moe, and Svein Arne Nordbø

Human bocavirus 1 (HBoV1) is a parvovirus associated with respiratory tract infections (RTIs) in children, but a causal relation has not yet been confirmed. To develop a qualitative reverse transcription PCR to detect spliced mRNA from HBoV1 and to determine whether HBoV1 mRNA correlated better with RTIs than did HBoV1 DNA, we used samples from HBoV1 DNA–positive children, with and without RTIs, to evaluate the test. A real-time reverse transcription PCR, targeting 2 alternatively spliced mRNAs, was developed. HBoV1 mRNA was detected in nasopharyngeal aspirates from 33 (25%) of 133 children with RTIs but in none of 28 controls (p<0.001). The analytical sensitivity and specificity of the test were good. Our data support the hypothesis that HBoV1 may cause RTIs, and we propose that HBoV1 mRNA could be used with benefit, instead of HBoV1 DNA, as a diagnostic target.

Human bocavirus 1 (HBoV1) is a small nonenveloped virus in the *Parvoviridae* family. It was discovered in human respiratory samples in 2005 (*1*). The virus does not grow in standard cell lines, and diagnosis has mainly been based on DNA detection with PCR. Detection of multiple viruses in HBoV1 DNA–positive airway samples from children with respiratory tract infections (RTIs) has been a characteristic finding in many studies (*2–4*). In addition, many healthy children have tested positive for HBoV1 DNA (*2*,*5*); thus whether the virus actually causes RTIs in children or is just a bystander to other infections has been debated. However, we have shown that the following 3 factors are associated with RTIs: HBoV1 viremia (HBoV1 DNAemia), a high HBoV1 DNA load in nasopharyngeal aspirates (NPAs), and monodection of HBoV1 DNA in NPAs (*5*). In addition, RTIs in HBoV1 DNA–positive children are associated with HBoV1 seroconversion (*6*). This evidence supports a causal relation between HBoV1 and RTIs in children, but DNA-based PCR tests do not seem to diagnose HBoV1 infection accurately. We propose that detection of HBoV1-specific mRNA, as a measure of actively transcribing virus, may be a better method.

Author affiliations: Trondheim University Hospital, Trondheim, Norway (A. Christensen, H. Døllner, L.H. Skanke, S. Krokstad, N. Moe, S.A. Nordbø); and Norwegian University of Science and Technology, Trondheim (A. Christensen, H. Døllner, N. Moe, S.A. Nordbø)

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The main objectives of this study were to develop a qualitative reverse transcription PCR (RT-PCR) detecting spliced mRNA from HBoV1 and to clarify whether HBoV1 mRNA detection may correlate better than DNA detection with RTIs in children. NPAs and blood samples from a group of children, with and without RTIs, who tested positive for HBoV1 DNA were used for this purpose.

Materials and Methods

Samples

HBoV1 DNA–positive NPA samples from an ongoing project on RTIs in children 0–16 years of age were used for evaluation of the test (*5*). In particular, 161 NPA samples collected at admittance from 161 children at the Department of Pediatrics, St. Olav's Hospital, Trondheim University Hospital (Trondheim, Norway), during June 2007–June 2010 were included. A blood sample was also available for 63 of the children. All samples had been stored at –70°C.

Children with RTIs

Of the 161 HBoV1 DNA–positive NPA samples, 133 were from children with RTIs. Median age was 17 months (range 3 months–5 years) and 60% were boys. They were classified as having either lower (86 children) or upper (47 children) RTI (LRTI; URTI). LRTI was diagnosed in the presence of dyspnea, signs of lower airway obstruction (wheezing, retractions), and/or a chest roentgenogram

with positive results (infiltrates, atelectasis, air trapping). URTI was diagnosed when rhinitis, pharyngitis, and/or otitis media was found without signs of LRTI. In addition, 3 children with RTIs, admitted during winter 2011–12, were followed up on 3 occasions, each over 2 months.

Children without RTIs (Controls)

Twenty-eight HBoV1 DNA–positive NPA samples were collected from a group of children who were admitted for elective surgery and who had exhibited no signs or symptoms of RTI during the previous 2 weeks. The children were included prospectively during the same period in 2007–2010. Median age was 31 months (range 15 months–6 years), and 70% were boys.

Tests for Other Respiratory Agents

All NPA samples from patients and controls were also tested with PCRs for adenovirus, coronavirus (OC43, 229E, and NL63), enterovirus, parechovirus, human metapneumovirus (HMPV), influenza A and B viruses, parainfluenza virus types 1–4, respiratory syncytial virus (RSV), rhinovirus, *Bordetella pertussis*, *Chlamydophila pneumoniae*, and *Mycoplasma pneumoniae*. The PCRs were in-house, real-time assays with TaqMan probes (Roche Diagnostics, Basel, Switzerland) (*5*). The analyses were conducted as part of the daily laboratory routine and performed within 24 hours after sample collection. The target for the HBoV1 DNA PCR was the nuclear phosphoprotein-1 gene. This PCR has been described (*4*). A semiquantitative approach was chosen, and a cutoff value of 10^6 copies/mL was used to distinguish between high and low HBoV1 DNA load in NPAs.

Spliced HBoV1 mRNA-PCR

We developed a real-time RT-PCR on the basis of TaqMan technology (Roche). The following primers were designed: forward 5′-CGGCGAGTGAACATCTCTG-GA-3′ (positions 203–223) and reverse 5′-TGCTT-GTCTTTCATATTCCCT-3′ (positions 2438–2418). The estimated PCR product spanned a spliced segment from positions 241 to 2236 of the complete genome for HBoV1 (GenBank accession no. NC007455), which gives a theoretical PCR product of 242 bp and an alternative product, including a short segment from positions 2044 to 2164, yielding a product of 363 bp (Figure 1). These estimations were based on in vitro studies performed by Chen et al. (*7*). The probe targeting the untranslated region upstream of the nuclear phosphoprotein-1 gene had the following sequence: 5′-FAM-TGTCCACCCAAGAAACGTCGTC-TAA-TAMRA-3′ (positions 2295–2319). The PCR for every sample was also run without reverse transcription to test for potential unspecific reactions with viral DNA. The theoretical PCR product from HBoV1 DNA would be 2,236 bp in length, which is too long for amplification by real-time PCR under normal conditions.

Total DNA and RNA were extracted by using NucliSens easyMag extractor (bioMérieux, Marcy l'Etoile, France), and reverse transcription was carried out with Universal RiboClone random primers (Promega, Fitchburg, WI, USA) and M-MLV Reverse Transcriptase (Life Technologies Corp., Carlsbad, CA, USA) at 37°C for 60 min. followed by 94°C for 10 min. The PCR was performed for 45 cycles at 95°C for 5 s., 55°C for 10 s, and 72°C for 20 s.

Amplification efficiency was calculated by using the formula $E=10^{(-1/S)}-1$, where S is the slope of the standard curve. A human DNA PCR (specific for the γ-glutamyltransferase light chain 1 gene on chromosome 20) was used as amplification control (*8*). Nucleic acid extract from a clinical sample positive for RSV was used as cDNA control. To make sure that mRNA had not been degraded during storage, we used an RT-PCR to detect human β actin mRNA (*9*).

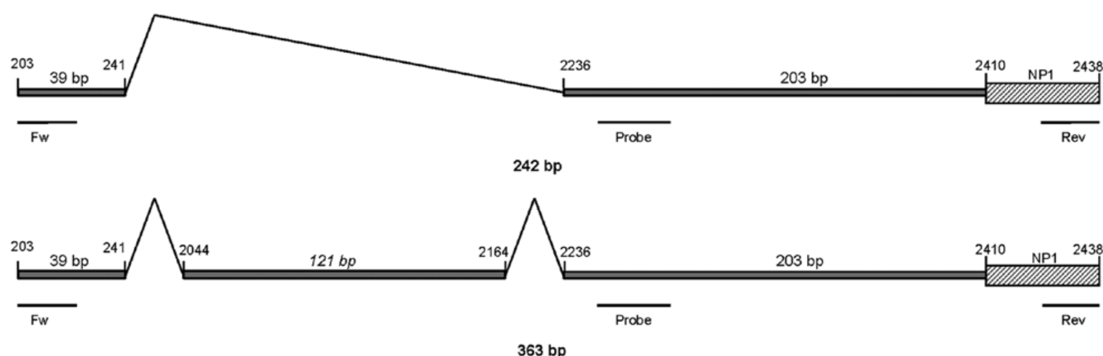

Figure 1. Schematic representation of the 2 human bocavirus 1 (HBoV1) mRNA PCR products, illustrating alternative splicing. Positions of primers and probe are shown. The total length of the upper product is 242 bp, and the length of the lower is 363 bp (reference sequence: GenBank accession no. NC007455).

RNA stability was studied by using clinical NPA samples and nucleic acid extracts from the easyMag extractor (bioMérieux). Four clinical NPA samples collected within the previous 2 hours were stored for 0, 1, 3, and 5 days at 4°C before nucleic acid extraction and testing with the HBoV1 mRNA PCR. One NPA sample was stored at room temperature and tested likewise. Two other clinical NPA samples were frozen and thawed 0, 1, 2, and 4 times before extraction and testing with the HBoV1 mRNA PCR (3 times was skipped to save NPA material). Furthermore, HBoV1 mRNA PCR results from 3 HBoV1-positive NPA samples stored at −70°C for 3 years were compared with nucleic acid extracts from the same samples stored under equal conditions for the same period. This was done to determine whether the stability of RNA in clinical NPA samples added to virus transport medium was comparable to the stability of RNA in nucleic acid extracts from the easyMag at this temperature. Relative changes in RNA load were measured by comparing logarithmically transformed cycle threshold values (Ct values) obtained from the same experiment.

Quantitative standards for the real-time HBoV1 mRNA PCR were made by cloning a plasmid (pCR4-TOPO; Life Technologies Corp.) containing the PCR product. The amount of nucleic acid was measured, and serial dilutions covering a range of 7 logs were made to measure the analytical sensitivity of the HBoV1 mRNA PCR.

Analytical specificity was evaluated by using cDNA from NPA samples positive for all respiratory agents included in the study. cDNA from NPA samples containing viruses that can be reactivated in the respiratory tract were also included (i.e., herpes simplex virus, cytomegalovirus, Epstein-Barr virus, and human herpes virus 6). Finally, cDNA from NPA samples positive for the more closely related parvovirus B19 and cDNA from fecal samples positive for human bocaviruses 2 and 3 (HBoV2 and HBoV3) were tested. The primers and probe described by Kantola et al. were used for detection of HBoV2 and HBoV3 (*10*). Two samples positive for each agent were used, and all samples had undergone extraction within 2–20 hours after sample collection. Sequence analysis on the PCR products was performed by using the BigDye Terminator Cycle sequencing method and the ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis

Statistical analysis was by χ^2 test for categorical variables and Student *t* test for continuous variables. Multiple logistic regression analysis was used to evaluate the association between detection of HBoV1 mRNA and LRTI, controlling for differences in age, sex, and the presence of other viruses among case-patients and controls. We report the odds ratio (OR) with 95% CIs and the corresponding p value as a measure of the strength of the association. All analyses were performed by using IBM SPSS Statistics version 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Spliced HBoV1 mRNA PCR

HBoV1 mRNA was detected in 33 of the 161 HBoV1 DNA–positive NPA samples (Table 1). Gel electrophoresis showed that primarily 2 PCR products were amplified with sizes of ≈250 bp and ≈400 bp (Figure 2). Sequence analysis showed that they were spliced products with either 1 or 2 introns cut out as expected (schematically illustrated in Figure 1). The exact product sizes were 242 bp and 363 bp. Direct PCR analysis for HBoV1 mRNA on the nucleic acid extracts, without initial reverse transcription, was negative for 30 of 33 NPA samples. For the remaining 3 samples, however, weak signals were detected. These 3 samples had very high HBoV1 DNA loads (range 4×10^8 copies/mL to $>10^{10}$ copies/mL), and were also strongly positive by the HBoV1 mRNA PCR after cDNA synthesis. The products of the 3 PCRs that were done without cDNA synthesis were sequenced. Product sizes were 145, 261, and 457 bp, and sequence analysis showed gaps at different positions, all of them lacking splice site characteristics (data not shown).

Amplification efficiency of the HBoV1 mRNA PCR was calculated on the basis of dilutions of both the nucleic acid extract and cDNA. It was measured to 100% in both cases, indicating a high efficiency of both the PCR and cDNA synthesis (data not shown). The assays' reportable range was from 500 copies/mL (10 copies/reaction) to 10^{10} copies/mL.

Results of the β-actin PCR performed after DNase treatment were positive for all samples studied. Results of the HBoV1 mRNA PCR were negative for all other respiratory agents, herpesviruses, and parvoviruses tested.

Table 1. HBoV1 mRNA PCR results in NPAs from children with and without RTIs, Norway, 2007–2010*

Sample source	Total no.	No. (%) HBoV1 mRNA$^+$	No. (%) HBoV1 mRNA$^−$	p value
Children with RTIs	133	33 (25)	100 (75)	p<0.001
Controls (without RTIs)	28	0	28 (100)	
Children with LRTIs	86	27 (31)	59 (69)	p = 0.02
Children with URTIs	47	6 (13)	41 (87)	

*HBoV1, human bocavirus 1; NPAs, nasopharyngeal aspirates; RTIs, respiratory tract infections; LRTIs, lower RTIs; URTIs, upper RTIs.

mRNA Stability

The HBoV1 mRNA load in NPA remained stable for 5 days at 4°C. At room temperature, it was unaltered after 24 h but was reduced by 1 log after 3 days and by 1.5 log after 5 days. Freezing and thawing of the NPA samples once or twice did not affect yield, but after 4×, it was reduced by ≈0.5 log. For 3 NPA samples that had been stored at −70°C for 3 years, the results were equal for both the nucleic acid extract and the original sample. HBoV1 mRNA PCR results for nucleic acid extracts were stable for weeks when samples were stored at 4°C (samples stored for up to 8 weeks were tested; data not shown).

Performance of Spliced HBoV1 mRNA PCR on Samples

First, we compared the rates of positive test results for HBoV1 mRNA among children with positive results for HBoV1 DNA, with and without RTIs. Only one fourth of the patients and none of the controls had test results positive for HBoV1 mRNA (Table 1). More children with LRTI (27/86 [31%]) than with URTI (6/47 [13%]) had positive test results for HBoV1 mRNA (Table 1). After we adjusted for age, sex, and presence of other viruses, this difference persisted (OR 3.5, 95% CI 1.3–9.8, $p = 0.02$).

We previously found that 3 factors (HBoV1 DNAemia, high HBoV1 DNA in NPAs, and monodection of HBoV1 DNA in NPAs) were each associated with RTIs in children (*5*). In the present study, these factors were strongly associated with a positive test result for HBoV1 mRNA (Table 2). The close relationship between HBoV1 DNA load and HBoV1 mRNA detection in NPAs is also illustrated in Figure 3. Of the 100 RTI patients who were negative for HBoV1 mRNA, 75 were positive for ≥1 other respiratory viruses. Twenty-eight (37%) of these children were infected with the highly pathogenic RSV. Distribution of the viruses most commonly co-detected with HBoV1 is shown in Table 3.

Follow up of 3 Children with RTIs during Winter 2011–12

During winter 2011–12, sequentially collected samples from 3 HBoV1 DNA–positive children made it possible to gain some information about changes in HBoV1 mRNA and HBoV1 DNA over time. One of these children (boy 1) was a 2-year-old boy with cerebral palsy who had been admitted with bronchiolitis. On admission, he had HBoV1 DNAemia and analysis of NPAs showed that he had 1) a high HBoV1 DNA load, 2) monodection of HBoV1 DNA, and 3) positive HBoV1 mRNA PCR results. He recovered slowly, and after 10 days a new NPA sample was taken. The HBoV1 DNA load was still high, but the results for the HBoV1 mRNA PCR were negative. Two months later, his NPAs still were positive for HBoV1 DNA and negative for HBoV1 mRNA. The other patients were two 1.5-year-old boys (boys 2 and 3), who had been included in an HMPV follow-up study. Their NPA samples became positive for HBoV1 DNA 1 week after HMPV infection was diagnosed. The initial samples were negative for HBoV1 DNA. The clinical condition was unaltered for both when HBoV1 DNA appeared. HBoV1 DNA loads reached moderate levels for boy 2 and high levels for boy 3, but for both boys, the PCRs were negative for HBoV1 mRNA in 2 consecutive NPA samples taken 5 days apart. Unfortunately, no blood samples were taken from these 2 boys.

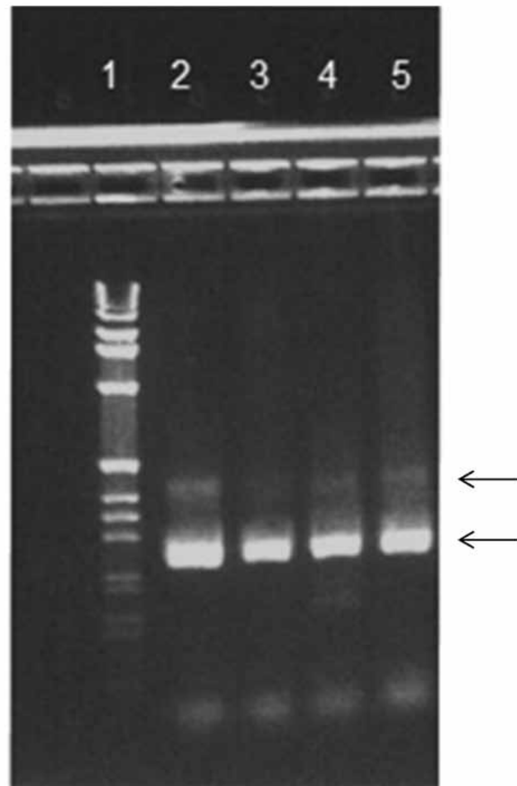

Figure 2. Agarose gel stained with ethidium bromide. Reverse transcription PCR products from 4 patients are shown in lanes 2–5 and 1-kb DNA Ladder (Life Technologies Corp., Carlsbad, CA, USA) in lane 1. Arrows indicate 2 bands corresponding to ≈250 and ≈400 bp.

Discussion

We report here the development of a robust PCR for detection of spliced mRNA from HBoV1. We found that HBoV1 mRNA correlated significantly better with RTIs in children than did HBoV1 DNA, indicating that this PCR may diagnose HBoV1 infection more accurately than PCR for HBoV1 DNA.

Table 2. HBoV1 mRNA PCR results in NPAs in relation to HBoV1 DNAemia, a high HBoV1 DNA load, and monodection of HBoV1 DNA, Norway, 2007–2010*

Factor	Total no.	No. (%) HBoV1 mRNA$^+$	No. (%) HBoV1 mRNA$^-$	p value
HBoV1 DNAemia, n = 63	17	13 (77)	4 (23)	p<0.001
No HBoV1 DNAemia	46	5 (11)	41 (89)	
HBoV1 DNA load, n = 161				
$\geq 10^6$ copies/mL	59	33 (56)	26 (44)	p<0.001
$<10^6$ copies/mL	102	0	102 (100)	
$\geq 10^8$ copies/mL	18	17 (94)	1 (6)	p<0.001
$<10^8$ copies/mL	143	16 (11)	127 (89)	
Monodection of HBoV1 DNA, n = 161	43	14 (33)	29 (67)	p = 0.022
Multiple virus detections	118	19 (16)	99 (84)	

*HBoV1, human bocavirus 1; NPAs, nasopharyngeal aspirates; HBoV1 DNAemia, HBoV1 viremia.

Splicing is a process specific for mRNA synthesis, and with use of a primer pair spanning an intron, spliced viral mRNA should be specifically detected within the frame of the familiar and robust RT-PCR. This diagnostic technique has been used to diagnose parvovirus in dogs and may also be an option for diagnosing HBoV1 infections in humans (*11*). Furthermore, detection of mRNA is routinely used for diagnosing human papillomavirus infections and has been studied for diagnosing human herpesvirus 6 and HIV infections (*12–15*). However, the mRNA tests for these viruses have been based on either specific mRNA extraction, nucleic acid sequence–based amplification technology, or pretreatment with DNase. The advantage with our approach is that no pretreatment, other than cDNA synthesis, is needed. The procedure is performed as a regular RT-PCR with standard equipment and will be easy to use in most routine laboratories. The analytical performance of the test was good with high analytical specificity and sensitivity.

The probe target was chosen to detect the 2 products (Figure 1) and thereby to maximize analytical sensitivity. A probe spanning the spliced segment between positions 241 and 2236 would have been an alternative approach, ensuring specific detection of mRNA spliced at this exact location (Figure 1). However, because our results indicated high specificity with the chosen probe, we did not develop this approach further.

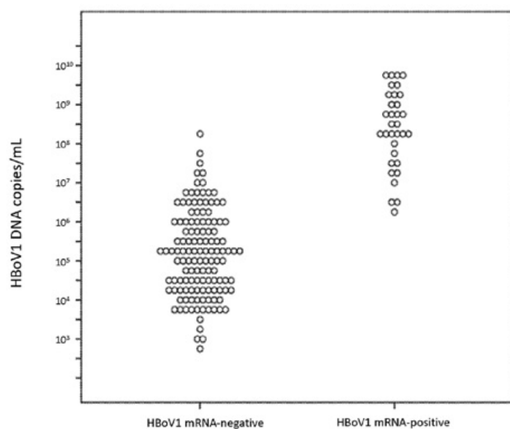

Figure 3. Distribution of human bocavirus 1 (HBoV1) DNA loads in nasopharyngeal aspirates either positive (n = 33) or negative (n = 128) for HBoV1 mRNA. Each dot indicates 1 sample.

Previously, RNA molecules were believed to have short half-lives because RNases may be present everywhere and easily degrade RNA. Recent studies, however, have suggested that mRNA may be stable when molecules are kept in the original biologic material (*16*,*17*). We found that the mRNA content in NPA samples was stable during a 5-day period at 4°C. This stability suggests that NPA samples kept in a refrigerator and processed within 1–2 days, which is standard in our laboratory, are safe to use for HBoV1 mRNA testing.

In 3 samples that had strong signals in the HBoV1 mRNA test, a weak signal was detected also without prior cDNA synthesis, evoking the question of whether HBoV1 DNA could give false-positive reactions in the HBoV1 mRNA test. The PCR was designed so that the theoretical DNA product would be 2,236 bp—too large for amplification to occur in a regular, real-time PCR. For this reason, the PCR products were expected to result from recombination events. Gel and sequence analysis showed that all 3 products had different sizes, ranging from 145 to 457 bp. Moreover, no common sequence profiles were found near the gap junctions, which seemed to be located at random. Homologous recombination, a concentration-dependent process, may explain this phenomenon because it occurred only in NPA samples with extremely high levels of HBoV1 DNA. We speculate that the PCR products might have been subpopulations of nonviable HBoV1 mutants that appeared when virus replication was at its highest. However, the specificity of the test was not affected because it happened only in patients with very high viral DNA loads and strong HBoV1 mRNA signals.

In addition to being a diagnostic test, this method may be used to gain information on HBoV1 transcription in vivo. Our data confirmed previous in vitro results on the splicing pattern at the 5′ end of the HBoV1 genome (Figure 1) (*7*,*18*).

Table 3. Most commonly co-detected viruses in NPAs from children with HBoV1 DNA, distributed by presence of RTI and HBoV1 mRNA, Norway, 2007–2010*

	HBoV1 DNA+ children with RTIs		HBoV1 DNA+ controls	
Virus	No. (%) HBoV mRNA+, n = 33	No. (%) HBoV1 mRNA–, n = 100†	HBoV mRNA+, n = 0	HBoV1 mRNA–, n = 28†
Respiratory syncytial virus	4 (12)	28 (28)	–	0
Rhinovirus	7 (21)	25 (25)	–	10 (36)
Enterovirus	5 (15)	24 (24)	–	15 (54)
Adenovirus	1 (3)	20 (20)	–	9 (32)

*NPAs, nasopharyngeal aspirates; HBoV1, human bocavirus 1; RTIs, respiratory tract infections; +, positive; –, negative.
†Triple and quadruple infections were common, and percentages within the columns may therefore add up to >100%.

For evaluation of the HBoV1 mRNA test, we were able to use available clinical samples from children with or without RTIs who had been tested for 18 respiratory agents and had HBoV1 DNA in NPAs (*5*). None of the children without RTIs had detectable HBoV1 mRNA. Because mRNA is a marker of active viral transcription, this finding indicates that the 28 asymptomatic children carried inactive HBoV1 or HBoV1 with low activity. The strong association found between active HBoV1 transcription and RTI in children supports the hypothesis that HBoV1 may cause RTIs. The hypothesis is further supported by the associations found between HBoV1 mRNA and the 3 factors: HBoV1 DNAemia, high HBoV1 DNA load in NPAs, and monodection of HBoV1 DNA—all factors strongly related to RTIs in children (*5*,*6*,*19*). In addition, the fact that HBoV1 mRNA was more frequently detected in children with LRTIs than with URTIs indicates that LRTI is a prominent manifestation of HBoV1 infection.

Only one fourth of the HBoV1 DNA–positive children with RTIs had detectable HBoV1 mRNA. Similar findings were recently reported by Proenca-Modena et al. (*20*). The absence of HBoV1 mRNA in most of the children with RTI may indicate that these children did not have a clinical HBoV1-infection, despite positive test results for HBoV1 DNA. Indeed, other respiratory viruses were frequently detected among the children who had a negative HBoV1 mRNA test result; RSV accounted for one third of infections.

The previously mentioned strong relation between HBoV1 DNA load in NPAs and HBoV1 mRNA is illustrated in Figure 3. It shows 2 distinct populations with little overlap, and good discrimination between HBoV1 mRNA–positive and –negative samples can be achieved with cut-off values from 10^6 to 10^7 HBoV1 DNA copies/mL. We suggest that, for clinical purposes, HBoV1 mRNA is more accurate than HBoV1 DNA in diagnosing active HBoV1 infection, but a high HBoV1 DNA load (>10^7 copies/mL) may also be useful in diagnosis.

Previously, HBoV1 has been found to persist in NPAs for many months (*21*–*23*). The molecular basis for this persistence is largely unknown, but 2 recent studies have given evidence in support of persistent circular HBoV episomes (*24*–*26*). The NPA samples in our study which were negative for HBoV1 mRNA and positive for HBoV1 DNA could be from patients with past HBoV1 infections who were still shedding viral DNA. Boy 1, who was followed up during winter 2011–12, may illustrate this. Results of PCR on NPA samples from this boy were positive for HBoV mRNA only for a short period (<10 days), coinciding with the acute symptomatic infection, whereas HBoV1 DNA persisted for months. An alternative hypothesis might be that the samples negative for HBoV1 mRNA and positive for HBoV1 DNA were from children with a latent HBoV1 infection. The findings in boys 2 and 3, who were followed up during the same winter, may support this hypothesis. HBoV1 DNA in NPAs appeared during an ongoing HMPV infection in both children, but results of PCR for HBoV1 mRNA remained negative in 2 consecutive samples. The lack of detectable HBoV1 mRNA may indicate that HBoV1 did not play a role in these infections. Release of latent HBoV1 DNA from cells disrupted by inflammation caused by HMPV may be a better explanation. More longitudinal studies, including serologic analyses, are needed to further study these relationships.

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Dr Christensen is a consultant in the Section for Virology, Department of Medical Microbiology, St. Olav's Hospital, and a doctoral candidate at the Institute of Laboratory Medicine, Children's and Women's Health, Norwegian University of Science and Technology, Trondheim. His research interests include viral respiratory infections, parvoviruses, picornaviruses, and paramyxoviruses.

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Address for correspondence: Andreas Christensen, Norwegian University of Science and Technology, Department of Laboratory Medicine, Children´s and Women´s Health, P.O. Box 8905, MTFS, 7489 Trondheim, Norway; email: a.christensen@ntnu.no