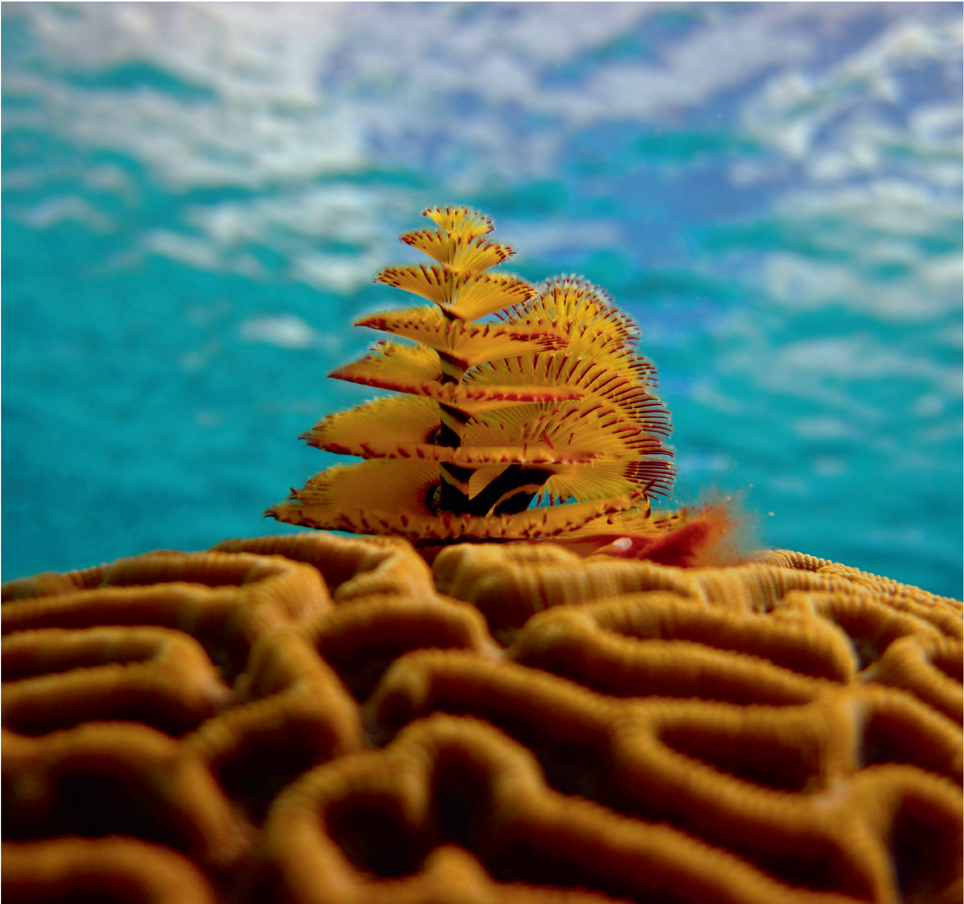
Human enteroviruses and parechoviruses: disease spectrum and need for treatment in young children



Joanne G. Simons-Wildenbeest

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Human enteroviruses and parechoviruses: disease spectrum and need for treatment in young children

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بنی آدم اعضای یک پیکرند
که در آفرینش ز یک گوهرند
چو عضوی به درد آورد روزگار
دگر عضوها را نماند قرار
تو کز محنت دیگران بی غمی
نشاید که نامت نهند آدمی

"Humans are limbs of one body
and are created with the same valuable essence.
When one limb passes its days in pain
the other limbs cannot remain at rest.
You, who feel no pain at the suffering of others
deserve not to be called human."

Saadi Shirazi, Persian poet

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Chapter 1

General introduction

Partly adapted from Kimberley S.M. Benschop, Joanne G. Wildenbeest, Dasja Pajkrt and Katja C. Wolthers (2012). Human Parechoviruses, New Players in the Pathogenesis of Viral Meningitis. In George Wireko-Brobby (Ed.), Meningitis. ISBN: 978-953-51-0383-7, InTech

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The Picornaviridae

Picornaviruses are among the most prevalent viruses in humans and animals. In humans they are able to cause a wide variety of disease ranging from the common cold to life threatening infections like myocarditis and meningoencephalitis.
Picornaviruses are small, non-enveloped single-stranded RNA viruses. The *Picornaviridae* family nowadays consists of 26 genera of which 7 genera are known to infect humans: *Enterovirus, Parechovirus, Hepatovirus, Cardiovirus, Cosavirus, Kobuvirus* and *Salivirus*.[1] Enteroviruses, hepatoviruses and parechoviruses are the most prevalent and clinically relevant picornaviruses in humans. The *Hepatovirus* genus consists of only one species and one serotype that can infect humans (hepatitis A virus (HAV)), giving symptoms of (self-limiting) acute hepatitis. Vaccination with an inactivated vaccine is highly efficacious in preventing clinical disease. In addition, passive immunisation with HAV specific immunoglobulins is available for young children and as post-exposure prophylaxis.[2]
The *Enterovirus* genus consists of several human species (human enterovirus (EV) A-D, human rhinovirus (HRV) A-C) and multiple (sero)types.[1] The disease spectrum varies widely from asymptomatic or mild disease to severe infections like meningoencephalitis and myocarditis. Most infections are self-limiting, but in the case of severe infection, treatment options are very limited since there is currently no effective anti-enteroviral drug available. Except for poliovirus (species EV-C) no vaccine is available. The *Parechovirus* genus contains two species: Ljungan virus and human parechovirus (HPeV). Ljungan virus consists of 4 serotypes and was first detected in bank voles.[3] Ljungan virus is also frequently seen in rodents.[4] Although a relation with disease in foetuses and infants was suggested,[5,6] this remains controversial and has never been proven.[7] The HPeV species is only found in primates and now consists of 16 types.[1] The disease spectrum is similar to that of EV infections, although HPeV infection is almost exclusively seen in young children. No antiviral drugs or vaccines are available.

Genome structure of enterovirus and parechovirus

Both EV and HPeV have a positive sense, single-stranded RNA genome consisting of around 7400 nucleotides (Figure 1).[8] A 5'untranslated region (5'UTR) of ~700 nucleotides precedes the single open reading frame of ~6600 nucleotides encoding a single polyprotein. This is followed by a small 3'UTR of 70-80 nucleotides and a poly(A)tail. The polyprotein consists of three regions (P1-P3). P1 encodes the structural region and is cleaved in the viral capsid proteins VP0, VP1 and VP3. The P2 and P3 regions encode the non-structural proteins 2A-C and 3A-D, which are involved in replication and host-cell interaction functions. In EVs the VP0 capsid protein is cleaved into VP4 and VP2 during maturation, resulting in 4 structural proteins. In HPeVs the VP0 capsid protein is not cleaved, resulting in only 3 structural proteins. The VP proteins form an icosahedral capsid of ~30 nm (Figure 1).

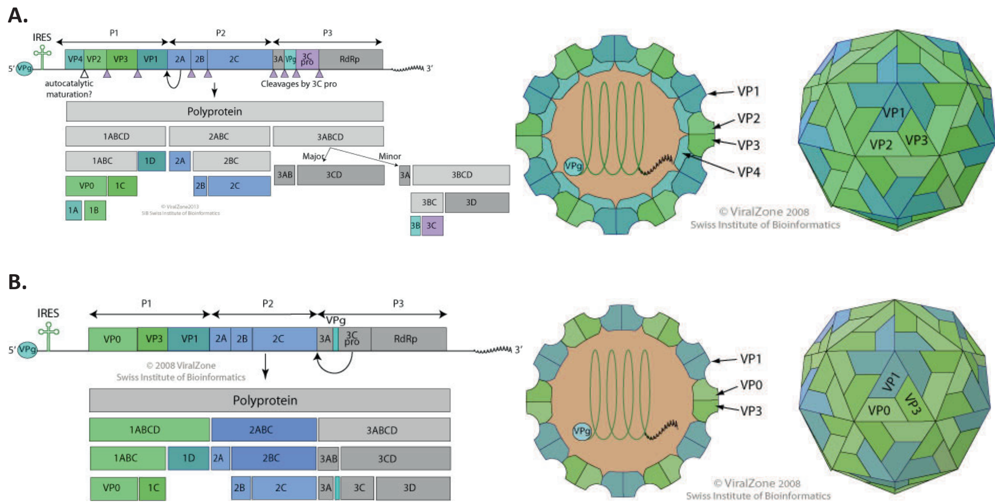

Figure 1. Genome organisation of human enterovirus **(A)** and human parechovirus **(B)**. Reprinted with permission from ViralZone, SIB Swiss Institute of Bioinformatics.

Disease spectrum and classification

Enterovirus

One of the most well-known human EVs is poliovirus (3 types), which was discovered in the 1940s (reviewed in Melnick *et al.*[9]). Subsequently the species Coxsackievirus (CV), later divided in CV-A and CV-B, was described for the first time in 1948.[10] With newer techniques the species Enteric Cytopathogenetic Human Orphan (ECHO) virus was added. Since 1974 newly identified human EVs were no longer classified in the above-mentioned species, but were assigned a number.[11] With the development and widespread use of molecular techniques, the old classification was not adequate anymore and types were reclassified into the species A-D (Table 1).[1]

Table 1. Reclassification of human enteroviruses into Enterovirus A-D.

	Enterovirus A	Enterovirus B	Enterovirus C	Enterovirus D
Poliovirus			1-3	
Coxsackie A virus	2-8, 10, 12, 14, 16	9	1, 11, 13, 17, 19-22, 24	
Coxsackie B virus		1-6		
ECHO virus		1-7, 9, 11-21, 24-27, 29-33		
Enterovirus	71, 76, 89-91, 114, 119-121	69, 73-75, 77-88, 93, 97, 98, 100, 101, 106, 107, 111	95, 96, 99, 102, 104, 105, 109, 113, 116-118	68, 70, 94, 111

Poliovirus has been associated with large outbreaks of acute flaccid paralysis with an impressive morbidity and mortality in especially children. It is the only human EV against which an effective vaccine has been available since the 1950s, resulting in an almost worldwide eradication of poliovirus. However, despite the efforts of the Global Polio Eradication Initiative that was started in 1988 by the World Health Assembly, with the aim to eradicate polio using the life attenuated oral polio vaccine (OPV), polio is still endemic in 3 countries (Afghanistan, Nigeria and Pakistan).[12] In addition to local distribution problems related to political instability and suspicion of the local population about the purpose of vaccination, the emergence of vaccine-associated paralytic poliomyelitis as a result of genetic reversion to neurovirulent strains is another challenge.[13,14] Prolonged circulation of circulating vaccine-derived polioviruses (cVDPVs) in areas with low vaccination coverage together with prolonged shedding of vaccine derived poliovirus in people with an impaired humoral immunity (immune-deficiency related vaccine-derived polioviruses (iVDPVs)) are other obstacles making global polio eradication more difficult to achieve. This was the reason for the World Health Organisation together with the Centers for Disease Control and Prevention to recommend a role for anti-polioviral agents in the combat against poliomyelitis.[15] These agents (preferably two agents administered simultaneously at least to prevent emergence of resistance) can be used to treat cases of acute poliomyelitis, to eradicate persistent shedding and circulation of cVDPVs and iVDPVs, and can be used in outbreaks as prophylaxis of exposed individuals.[15,16]

The non-polio EVs consist of more than 100 types causing a wide range of symptoms, from asymptomatic to mild respiratory and/or gastrointestinal infection, and more severe disease such as hand, foot and mouth disease (HFMD), meningitis, encephalitis, acute flaccid paralysis, pericarditis, myocarditis, hepatitis, pleurodynia and neonatal disseminated EV infection (reviewed in Tapparel *et al.*[11]). Although the different serotypes can overlap in the spectrum of disease, specific types can be related to specific disease. For example, CV-B is often associated with myopericarditis; echoviruses and CV-B are associated with meningitis and CV-A frequently causes HFMD.[2] The recently emerged EV71 mainly causes self-limiting HFMD, but may progress to severe neurologic disease like acute flaccid paralysis and brainstem encephalitis with cardiorespiratory dysfunction. Central nervous system (CNS) complications typically occur in (young) children. Since the late 1990s several outbreaks of massive EV71 infections with brainstem encephalitis and associated pulmonary edema caused hundreds of deaths in the Asian Pacific region (reviewed in Ooi *et al.*[17]). This has led to major efforts to find anti-enteroviral drugs and/or an effective vaccine, since neither were available. Recently, a phase III clinical trial with an inactivated (alum adjuvated) EV71 vaccine was conducted in China with promising results.[18-20]

HRVs were first discovered in the 1950s as cause of the common cold. HRVs differ from the other EVs and HPeVs because they do not survive in an acid environment like the gastric acid fluids. Their main site of infection and replication is therefore not the gastrointestinal tract, but the respiratory tract. HRV-A and -B consist of respectively 74 and 25 types (Figure

2). HRV-C has only recently been discovered by molecular techniques as they could not be cultured within the standard cell culture settings.[21] Nowadays at least 50 types are identified (Figure 2).

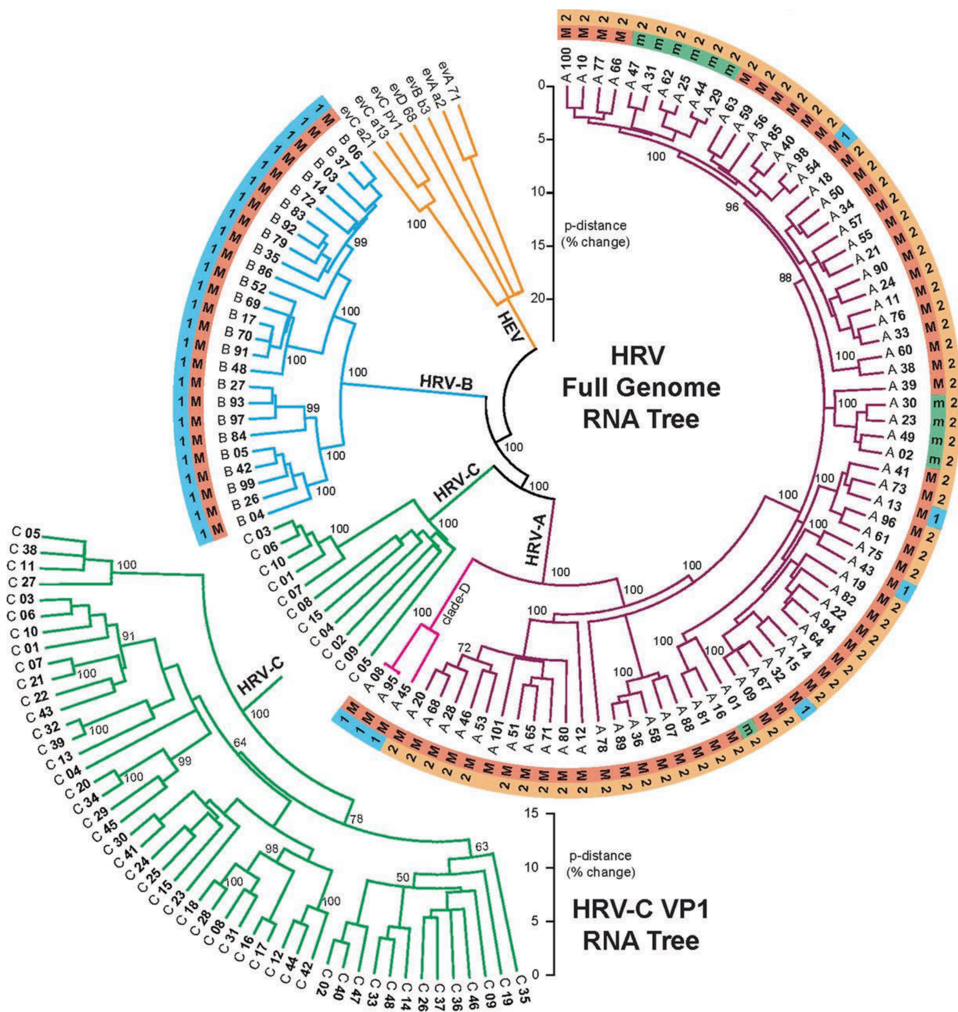

Figure 2. Phylogenetic tree of human rhinovirus types.
Reprinted from Knipe DM, Howley PM (ed), Fields virology, 6th ed. Wolters Kluwer Health/Lippincott Williams& Wilkins, Philadelphia, PA with permission from Wolthers Kluwer Health/Lippincott Williams&Wilkins.

HRV is the most common cause of upper respiratory tract infections (URTIs), found in more than half of the episodes of URTI.[22] Recently HRVs are identified as the second most common cause of bronchiolitis in hospitalized young children [23] and are associated with severe lower respiratory tract infections (LRTIs) in children admitted to the intensive care unit.[24] In addition, HRV-associated wheezing in the first 3 years of life is a risk factor for the development of asthma at the age of 6 years in a high risk cohort.[25] Several studies reported associations between HRV-C and asthma exacerbations,[26-28] lower respiratory tract infections [29] and more severe disease,[30] although other studies did not detect more severe disease in HRV-C infected children.[31,32] Moreover, asymptomatic HRV infections are also frequently seen in young children.[33,34]

Human parechovirus

Human parechoviruses were first discovered in 1956 during a summer diarrhea outbreak in the USA.[35] They were originally classified within the *Enterovirus* genus as ECHO virus 22 and 23. This was based on their biology in cell culture, exhibiting a similar cytopathogenic effect (CPE) as EVs, and their clinical presentation. With the introduction of molecular techniques, these viruses were reclassified as HPeV types 1 and 2 within the new genus *Parechovirus*.[36,37] Almost half a century after the discovery of HPeV1 and 2, a third HPeV type was discovered in Japan [38] and since then the number of HPeV types increased rapidly. Up to date there are 16 HPeV types known (Table 2).[1]

Table 2. HPeV prototype strains.[1]

Type	Strain	Origin	Reference
HPeV1A	Harris	Ohio, USA	Hyypia *et al.*, 1992 [39]
HPeV1B	BNI-788 St	Bonn, Germany	Baumgarte *et al.*, 2008 [40]
HPeV 2	Williamson	Ohio, USA	Ghazi *et al.*, 1998 [41]
HPeV 3	A308/99	Aichi, Japan	Ito *et al.*, 2004 [38]
HPeV 4	K251176-02	Amsterdam, the Netherlands	Benschop *et al.*, 2006 [42]
HPeV 5	CT86-6760	Connecticut, USA	Oberste *et al.*, 1998 [43]
HPeV 6	NII561-2000	Niigata, Japan	Watanabe *et al.*, 2007 [44]
HPeV 7	PAK5045	Badin, Pakistan	Li *et al.*, 2009 [45]
HPeV 8	BR/217/2006	Salvador, Brazil	Drexler *et al.*, 2009 [46]
HPeV 9	BAN2004-10902	Bangkok, Thailand	Oberste *et al.*, unpub.
HPeV 10	BAN2004-10903	Bangkok, Thailand	Oberste *et al.*, unpub.
HPeV 11	BAN2004-10905	Bangkok, Thailand	Oberste *et al.*, unpub.
HPeV 12	BAN2004-10904	Bangkok, Thailand	Oberste *et al.*, unpub.
HPeV 13	BAN2004-10901	Bangkok, Thailand	Oberste *et al.*, unpub.
HPeV 14	451564	Amsterdam, the Netherlands	Benschop *et al.*, 2008 [47]
HPeV 15	BAN-11614	Bangkok, Thailand	Oberste *et al.*, unpub.
HPeV 16	BAN-11615	Bangkok, Thailand	Oberste *et al.*, unpub.

Clinical symptoms of HPeV infections are generally similar to EV infections, ranging from mild respiratory and gastrointestinal disease to more severe disease like meningitis and sepsis-like illness (SLI). In earlier decades, when only HPeV1 and 2 were known, HPeV infections were considered of little clinical importance, even though occasionally severe disease was reported for HPeV such as acute flaccid paralysis, myocarditis, meningitis, encephalitis and encephalomyelitis.[48-52]

This perception of clinical presentations of HPeVs changed with the discovery of HPeV3.[38] HPeV3 infections were predominantly associated with neonatal sepsis and CNS infection (meningitis and encephalitis).[53-62] HPeV3 CNS infections account for approximately 3-17% of cases of meningitis or encephalitis in young children under 3 months of age,[44,54,63-66] ranking HPeV as the second dominant pathogen (after EV) of viral meningitis and encephalitis in young children.

HPeV1 is the most prevalent type, followed by HPeV3. HPeV4 is frequently found in stools,[47,67-70] while HPeV6 seems to prevail as a secondary respiratory pathogen.[71] Infections with HPeV2 and 5 are reported sporadically.[57,67,72] HPeV4, 5 and 6 have mainly been associated with mild gastrointestinal and respiratory symptoms in children, often with an underlying illness,[73] although recently 2 cases of neonatal sepsis caused by HPeV4 were described in Finland.[74] Circulation patterns of the newly reported HPeV types 7–16 are yet to be determined.[47,57,67]

While EVs generally affect individuals of all ages, more than 90% of HPeV infections have been described in children younger than 5 years of age.[40,47,67,75-81] Remarkably, the median age of children infected with HPeV3 is significantly lower than the median age of children infected with HPeV1,[53] with the majority of HPeV3 infections occurring in neonates and children under the age of 2 months.[44,47,54,58,61,63,65,82-84] This age difference in relation to the difference in disease severity between the two HPeV types suggests that neonates in comparison to older children might be less protected against HPeV3 infection. Most HPeV1 infections are presumed to occur within the first year of life, following the decline in circulating maternal antibodies. Seroprevalence data showed that 95-99% of neonates were boosted with antibodies against HPeV1 which are most likely from maternal origin.[85-89] This high HPeV1 seroprevalence suggests that the majority of infants are supposed to be protected from HPeV1 infection early in life via maternal antibodies. However, this may not always be the case as suggested by Ehrnst *et al.*[90] The HPeV1 seroprevalence decreases in the first 6 months of life, only to rapidly increase to 95% in children older than 1-3 year.[85,89] The low seropositivity from 6 months to 1-3 years is marked by an increase in infection frequencies among children in this age group.[53,85,87-89]

For HPeV3, the seroprevalence is approximately 70% among adults in Japan.[38] The lowest seroprevalence rate (15%) was seen in children between 7-12 months and steadily increased to 91% in adolescence only to decline again to 56-87% in adulthood. A recent study showed that seroprevalence among adults in Europe is only 10-13%, and even lower in children (3%).[91] This is in contrast to what is seen for HPeV1 seroprevalence: >90% of adults have

antibodies against HPeV1.[85,87,89,91] This may indicate that children are less protected through maternal antibodies specifically for HPeV3, explaining the young age and increased disease severity of HPeV3 infected children in comparison to HPeV1 infected children.

Diagnosis

Classically, HPeVs and EVs can be diagnosed through cell culture isolation, usually involving monkey kidney cells and human fibroblasts.[67,92] Other cell lines, such as the HT-29 (human colon adenocarcinoma), A549 (human lung carcinoma) and RD (rhabdomyosarcoma) cell lines can be used for culturing HPeV isolates as well.[44,67,75,93] However, cell culture has its limitations and CPE produced by HPeVs is not significantly different from the CPE elicited by EVs resulting in misidentification of HPeVs as EVs in the laboratory settings in which specific serotyping is not readily available.[53] This also explains the original classification of HPeVs as EVs.[35] HPeV types other than HPeV1 and 2 cannot be serotyped because specific antibodies are not readily available (HPeV3-6), or because they cannot be cultured at all (HPeV7-14). HRVs are difficult to culture as well and grow best in human fetal embryonic lung fibrobast cell lines and certain HeLa cell clones.[94,95] CPE appearance is very similar to EVs and can be distinguished by acid stability testing; HRVs are destabilized in an environment with low pH such as the gut while EVs are relatively resistant (reviewed in Jacobs *et al.*[95]).
In recent years polymerase chain reaction (PCR) became the state-of-art test to detect EVs in different patient materials. Most real-time RT-PCRs target the 5'UTR region, which is highly conserved among all EVs and HRVs. A problem is cross-reactivity between HRV and EV, making differentiation sometimes difficult.[95] PCR specific for EVs will fail to detect HPeVs because the targeted 5'UTR is too diverse between HPeVs and EVs.[53,96-99] Therefore a separate real-time RT-PCR specifically targeting the 5'UTR of HPeVs has been developed and validated for HPeV detection in CSF, blood, stool and respiratory samples.[40,47,81,100-103] Genotyping is increasingly used instead of serotyping to differentiate between the different species and types. By targeting the variable capsid region VP1 or VP1/VP3, HPeV and EV positive samples can be genotyped directly from clinical material.[54,58,67,104] For genotyping of rhinoviruses the VP1 and/or VP4/VP2 region are commonly used.[95,105]

Transmission

While HRV is thought to be mainly spread from person to person by aerosols,[106] the transmission route of EV and HPeV is usually fecal-oral through direct person to person contact or through ingestion of contaminated food or water (indirect transmission). Surface water can get contaminated with HPeV and EV easily because these viruses are shed in high amounts in stools and concentrations remain relatively high, even in treated sewage

water. In addition, these viruses are able to persist in the environment for several weeks to months.[107,108] Various outbreaks of recreationally associated waterborne disease by EVs have been reported (especially in children), but these are probably only the tip of the iceberg (reviewed in Sinclair *et al.*[109]).

Immune response

Most of what we know from picornavirus immunity is distilled from immunological studies with EV infections. In contrast with most viruses against which T cell dependent immune responses are of importance, an efficient host response against picornaviruses is considered to be mainly dependent on a proper humoral immune response with release of neutralizing antibodies (nAbs). After contact with an antigen, B lymphocytes are activated to form plasma cells. Plasma cells will subsequently produce antibodies which will neutralize the antigen. Part of the B cells will transform into memory cells. These memory cells can react quickly and release antibodies if the antigen is encountered again. The immunoglobulins produced by these plasma cells are mainly immunoglobulin G's (IgGs). Maternal IgGs are transferred through the placenta, protecting neonates and young infants from infection. These maternal IgGs are of particular importance in protection against disease in the first 3-6 months of life. After 3-6 months maternal antibodies are waning and children have to rely on their own immune responses. The important role of the humoral immunity is underlined by the increased incidence of severe EV infections in patients with primary antibody deficiency (PID), such as X-linked agammaglobulinemia (XLA), in which chronic enteroviral meningoencephalitis (CEMA) is one of the most severe complications.[110,111] Successful treatment with therapeutic immunoglobulin therapy (e.g. intravenous immunoglobulin (IVIG)) in PID patients with an EV meningoencephalitis provides additional evidence for an important role of nAbs for an adequate immune host response in severe EV infections. In addition, in neonates, lack of specific maternal EV antibodies is shown to be a risk factor for the development of severe illness.[112]
Knowledge of the host immune response to HPeV is in comparison to EV even more limited. In contrast to the evidence as described in the section above, there are no data available on the protective role of nAbs in HPeV infections. Seroprevalence of HPeV1 in adults is high (>95%) and HPeV1 infection in children is generally seen above the age of 6 months, suggesting that maternal nAbs protect young infants against HPeV1 infection.[89] In addition, the lower seroprevalence of HPeV3 in adults [38,91] in combination with the younger age at which HPeV3 infection occurs, might suggest a lack of maternal protection against HPeV3 in the early months of life.
The role of the innate immune response against *Picornaviridae* was historically considered of no importance and received little attention in the field of immunological research. However, in recent years the importance of the innate immune response, especially Toll-like receptors

(TLRs), against picornaviruses is more and more recognized. TLRs are transmembranic glycoproteins that are expressed on various cells types. There are 10 TLRs recognized in humans so far; TLR1, -2,- 4, -5, -6 are expressed on the cell surface sensing mainly bacterial products while TLR3, -7, -8 and -9 are located intracellular in vesicles and are activated by intracellular nucleic acids. Once activated, TLRs induce inflammatory responses by enhancing the production of various cytokines (reviewed in Beutler *et al.* [113] and Kemball *et al.*[114]). TLR7 and TLR8 seem to be of importance in the immune response against EVs, HRVs and HPeVs.[115-117] In addition TLR3 and TLR4 are triggered by CV-B infections,[118,119] while TLR2 recognizes HRV6.[117] The inflammation produced by enhanced expression of TLR8 seems to play a major role in the pathogenesis of dilated cardiomyopathy caused by CV-B.[120] However, the exact mechanisms how TLRs and other parts of the innate immune system influence the host response against EVs and HPeVs remains to be elucidated.

Treatment

There is no antiviral treatment against EVs and HPeVs currently available. Despite decades of research on anti-picornavirus medication, none of the drugs was licensed for use in patients. Most effort was made to find a drug against EVs. Only pleconaril was tested in phase III clinical trials. Pleconaril inhibits viral replication by integration into the hydrophobic pocket inside the viral capsid. As a result, the virus capsid is rigidified and in several cases the uncoating and binding of the virus to the host cell are interrupted.[121] The hydrophobic pocket is relatively well preserved among EVs and HRVs, resulting in a broad-spectrum anti-enteroviral and anti-rhinoviral activity of pleconaril. However, EV71 is not susceptible for pleconaril.[16] Since the capsid of HPeVs is different,[87,122] suggesting that the hydrophobic pocket differs from that of EVs, it is not likely that pleconaril has any activity against HPeV.[123] The US Food and Drug Administration (FDA) rejected pleconaril for the treatment of common cold because of the risk of side effects. Meanwhile pleconaril was used on compassionate use basis in immunocompromised patients with severe or chronic enteroviral infections with various outcomes. The drug was never licensed for this indication. Now, the drug is no longer available, although 2 clinical trials were conducted recently; one trial studied the effect of pleconaril nasal spray on the occurrence of rhinovirus associated common cold and asthma exacerbations in children >6 years and adults (NCT00394914 [124]). The other study was a double-blind, placebo-controlled, virologic efficacy trial of pleconaril as treatment for neonates with enteroviral sepsis syndrome (NCT00031512 [124]). The results of both these trials have not been published yet.

The major problems with anti-enteroviral treatment are that the *Enterovirus* genus is very diverse with many serotypes, therefore a drug with broad–spectrum antiviral activity is needed. Furthermore, the mutation rate in picornaviruses is relatively high, resulting in a high risk of selecting drug resistant strains.

Nowadays supportive treatment and administration of IVIG are the only available options for treatment of severe EV and HPeV infections. IVIG is haphazardly given to neonates and children with severe disease like myocarditis to reduce disease burden from EV infection, although its efficacy has not been proven. A randomized trial in neonates indicated that IVIG with a high nAb titer against the infecting EV type resulted in faster clearance of viremia. However, no effect on clinical outcome was demonstrated (possibly due to the small sample size).[125] The use of IVIG in EV71 outbreaks was evaluated retrospectively and showed a beneficial effect when given early in the course of the disease.[126,127] This was supported by high titers of EV71 specific nAbs found in Chinese donors, although a randomized controlled trial was never conducted.[128] Evaluation of effectivity of IVIG is also complicated by the observation that EV nAb titers in IVIG vary between batches produced in various geographic regions.[128-130]

Outline of this thesis

The aim of this thesis is to describe the disease spectrum of picornavirus infections in children (including EV, HPeV and HRV) and to assess the clinical relevance of an infection detected in the era of new and sensitive molecular diagnostic tools (PCR) which are now widely routinely used in clinical settings. The second aim is to evaluate the need for treatment against these infections, the available treatment options and the role of neutralizing antibodies as potential therapeutic options.
Part one focuses on the clinical relevance of HPeV and HRV-C infections. In contrast to the EVs, which are well known to cause significant morbidity, these species have only recently been discovered and their disease spectrum is not yet fully established. Despite the increasing number of studies on disease caused by HPeV, the clinical relevance of HPeVs (especially in stool samples) is still under debate. In **chapter 2** the prevalence of HRV infections in an unselected birth cohort is described and clinical symptoms are compared with HRV negative children and between HRV species A, B and C infected children. In **chapter 3** the clinical relevance of a positive HPeV1 and 3 PCR in stool samples is discussed and differences in clinical characteristics between HPeV1 and 3 are described. In **chapter 4** the duration of HPeV shedding in stools after symptomatic infection is described and related to viral load and clinical symptoms. The role of environmental (water) exposure in the occurrence of HPeV and EV infections is studied in **chapter 5**.
In part two treatment options for EV and HPeV infections are discussed. **Chapter 6** gives an overview of (the lack of) treatment options for severe HPeV infections. This is compared with the available treatment options for severe EV infections. In **chapter 7** treatment with pleconaril and IVIG in 2 patients with agammaglobulinemia and chronic enteroviral meningitis is described and compared to *in vitro* susceptibility of the EV types. **Chapter 8** describes the characteristics of the natural pleconaril resistant echovirus 11 strain found

in chapter 7 and possible mechanisms how this resistance could have been evolved. The successful treatment with IVIG of an infant with HPeV1 associated dilated cardiomyopathy and relation with HPeV1 specific neutralizing antibody titers in IVIG is described in **chapter 9**. **Chapter 10** describes specific cell tropism and neutralization characteristics of HPeV1 and 3 and implications for therapy development. In **chapter 11** the relation between (severity of) HPeV infection in infants and maternal antibodies is studied as part of the PARMA-study (PARechovirusinfections and Maternal Antibodies study). The aim of this study was to provide a rationale for specific antibody therapy in severe HPeV and EV disease.
In part three the results of this thesis are summarized (**chapter 12**) and discussed (**chapter 13**) and put in perspective of the current knowledge.

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PART I

Clinical relevance

Chapter 2

Rhinovirus C is not associated with wheezing or severe disease in an unselected birth cohort from the Netherlands

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Submitted

Abstract

Background

Human rhinovirus (HRV) is a frequent pathogen in young children, eliciting symptoms ranging from common colds to wheezing illnesses and lower respiratory tract infections. The recently identified HRV-C seems to be associated with asthma exacerbations and more severe disease, but results vary. We studied the prevalence and severity of infection with HRV in an unselected birth cohort.

Methods

Children with respiratory symptoms entered the symptomatic arm of the cohort and were compared to asymptomatic children. Severity of wheezing and other respiratory symptoms were registered and respiratory viruses were evaluated using throat and nasopharyngeal swabs on first presentation and after recovery (wheezing children). HRV genotyping was performed on HRV-PCR positive samples.

Results

HRV was the most prevalent respiratory virus and was found in 58 of 140 (41%) symptomatic children, in 24 of 96 (26%) control children and in 19 of 74 (25%) symptomatic wheezing children after recovery ($p<0.05$). HRV-A was the most commonly detected type (59%) followed by HRV-C (32%) and HRV-B (9%). Children with HRV mono-infection had more severe symptoms, but HRV infections were not associated with occurrence of wheezing. There was no association between the different HRV species and occurrence of wheezing or severity of disease. Symptomatic HRV-PCR positive children, in particular wheezing children, had a significant higher viral load than asymptomatic children.

Conclusions

In an unselected birth cohort from the Netherlands, HRV was the most prevalent respiratory virus. Our results suggest that HRV-C is not associated with more severe disease or wheezing in young children in the general population.

Introduction

Human rhinovirus (HRV) infections account for most respiratory infections in early life, being a major contributing factor to childhood morbidity (reviewed in Kieninger *et al.*[1]). Furthermore, episodes of HRV-induced wheezing are strongly associated with the subsequent development of asthma in high risk children.[2]

HRV belongs to the genus *Enterovirus* in the family of *Picornaviridae*. There are over 100 serotypes which are classified into 3 species; A, B and C.[3]

HRV infections in childhood cause a wide variety in clinical presentations ranging from very mild 'common cold' symptoms to severe life-threatening lower respiratory tract infections (LRTI).[3] Using novel molecular detection techniques, HRVs were identified as a common cause of bronchiolitis,[4] wheezing[5] and pneumonia.[6,7]

This spectrum of variation in clinical presentation is subject of ongoing research. Firstly, evidence suggests that symptomatic HRV infections reveal an underlying predisposition to develop asthma which may be modulated by genetic host factors.[8] On the other hand, HRV may also play a causal role in the development of asthma through promoting exaggerated inflammation and airway hyper-responsiveness.[9] Associations between varying clinical severity and different HRV serotypes favor evidence in support of a causal role for HRV in the onset of asthma. More specifically, the recently identified HRV-C[10] was found to be present in the majority of children admitted to the hospital with wheezing or acute asthma exacerbations and HRV-C infections were associated with increased severity of those exacerbations.[11-13] Furthermore, HRV-C was reported as the only species more frequently associated with lower respiratory tract infections in children as compared to the adult patient population.[14] By contrast, asymptomatic HRV-C infections have also been reported in healthy controls.[15]

As suggested by a recent review from Kieninger *et al.*[1], longitudinal studies on occurrence of both symptomatic and asymptomatic HRV infections in an unselected population will increase our knowledge on whether clinical manifestations of HRV infections are related to a predisposed host immune response or related directly to viral pathogenicity.

In this study, we hypothesized that the presence of HRV-C is associated with more severe acute respiratory infections in pre-school children from an unselected birth cohort. We studied this by comparing prevalence, clinical symptoms (specifically wheezing) and viral loads of different HRV types in HRV positive and negative symptomatic children. These prevalences were compared to the prevalence of HRV infections in asymptomatic controls and of the symptomatic children after recovery from wheezing respiratory illnesses. This recovered group of wheezing children is of specific interest because they have a high-risk phenotype for the development of asthma later in life.[2]

Methods

Subjects

This study is part of the EUROPA-trial (Early Unbiased Risk Assessment of Pediatric Asthma), a prospective cohort study in the Netherlands, focusing on prediction of early signs of asthma. Participants were recruited by targeted mailing from an unselected birth cohort of 12.033 infants born in greater Amsterdam and aged between 0 and 12 months old at inclusion. Exclusion criteria were a gestational age of less than 31 weeks or the presence of any manifest illness at inclusion, specifically any pulmonary disorder. A total of 1216 infants were included into the trial after both parents provided consent (Figure 1). At inclusion a structured baseline questionnaire was obtained.

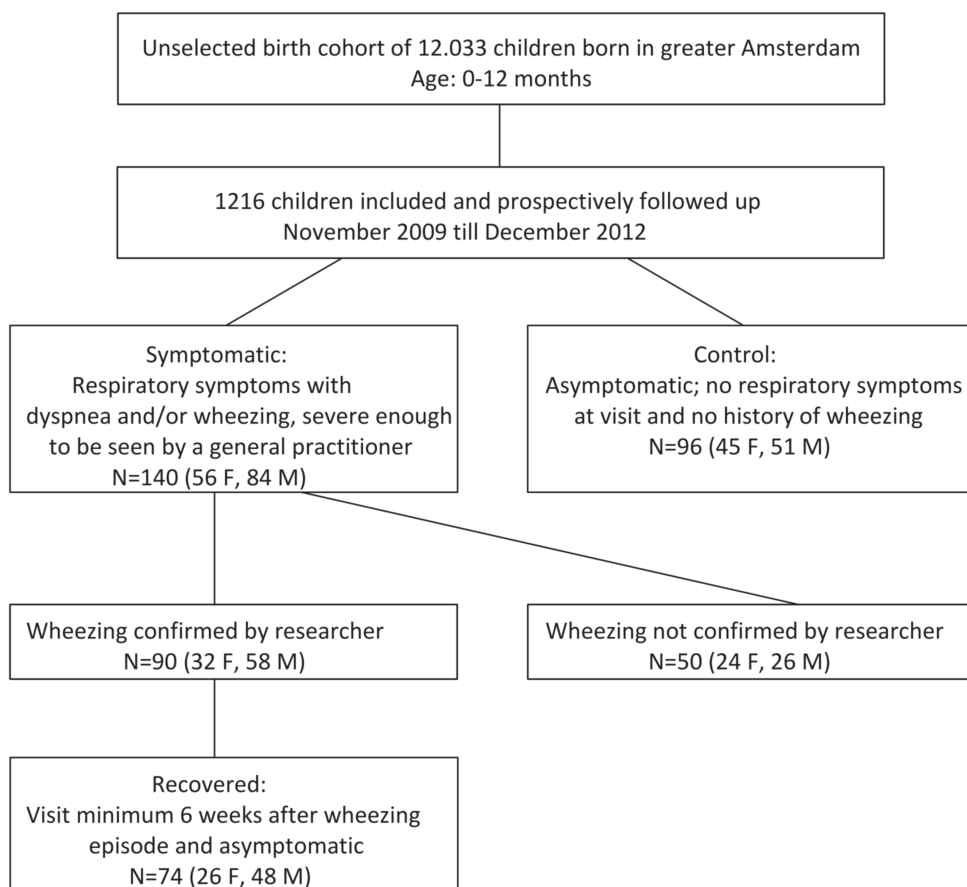

Figure 1. Selection process of children of the EUROPA birth cohort.

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Design

This study was designed as a prospective case-control follow-up study. Participating parents were instructed to contact the study team whenever their infant experienced respiratory tract symptoms from November 2009 until December 2012. A standardized telephone interview was obtained to assess the presence of symptoms. Infants experiencing cough, wheezing, labored breathing and/or dyspnea sufficiently severe for parents to warrant a visit to their family physician entered the symptomatic arm of the study and were visited by the study team within 8 hours after establishing these symptoms.

During the visit the presence and severity of acute respiratory symptoms was assessed by both parents and the on-site researcher. The researchers were well trained to recognize wheezing. The intra-class correlation was validated by means of evaluation of tracheal sound recordings by 5 pediatric pulmonologists in the first 30 patients which reached a Cronbach's alpha of 0.75. The study team assessed symptom severity (physician symptom score) by scoring the presence of suprasternal retractions, scalene muscle contraction, air entry and wheezing by auscultation, all part of the Pediatric Respiratory Assessment Measure.[16] Values of the physician symptom score range from 0 to 10 with increasing values indicating increased severity. Parents self-assessed severity by Asthma Control Questionnaire (ACQ)[16] modified for by proxy use (mACQ), although this questionnaire has not been validated for this application.

For our secondary aim we assessed the prevalence of HRV infection in asymptomatic children by recruiting controls from the same cohort who had never experienced lower respiratory tract symptoms severe enough to contact their family physician. If lower respiratory tract symptoms still occurred after being visited as a control a novel control was recruited.

Furthermore, children who were evaluated for an episode of respiratory symptoms in this study and were found to have viral induced wheezing were re-assessed after a symptom free period of at least 1 week, minimally 6 weeks after their initial presentation. Symptom free was defined as a physician and parent (ACQ-based) severity score of 0.

This study was approved by the Medical Ethical Committee of the Academic Medical Center Amsterdam (09/066) and the parents gave written informed consent. The EUROPA study is registered in the Dutch Trial Register (NTR-1955).

Virological analysis

At each visit the study team obtained naso- and oro-pharyngeal swabs (Copan Swabs, Brescia, Italy). The collected naso- and oro-pharyngeal samples were assessed for the presence of respiratory associated viruses *(HRV, human enterovirus (EV), human parechovirus (HPeV), influenzavirus A and B, para-influenzavirus 1, 2, 3 and 4, human bocavirus (HBOV), human coronavirus, respiratory syncytial virus, adenovirus and human metapneumovirus)*, using a multiplex PCR as described previously by Jansen *et al.*[17] A Ct-value of 40 or more was considered to be negative.[18]

HRV typing

HRV RNA was extracted from 200 µl HRV-positive sample with the MagnaPure LC instrument® using the total nucleic acid isolation kit (Roche Diagnostics). Genotyping was performed by amplifying a 540-base pair fragment spanning part of the 5'-untranslated region (UTR), capsid protein VP4 and part of VP2 (VP4/VP2) of the HRV-genome using a two-step semi-nested protocol.[19]

First, 6 µl of RNA was reverse transcribed and amplified with the SuperScript III one-step RT/Platinum Taq polymerase kit (Invitrogen) according to the manufacturer's instructions using primers adapted from Savolainen *et al.*[20] (shown in Table 1) and cycling conditions described by Harvala *et al.*[21]

One µl of the combined RT-PCR product was then used as input for the second semi-nested PCR amplification. The reaction mix contained 1x PCR buffer, 2.5 mM $MgCl_2$, 0.5 µM of each primer, 200 µM of each dNTP, 0.1 µg/ml BSA, and 0.05 U of FastStart Taq polymerase (Roche) in a 20 µl-reaction volume. Cycling conditions were as follows: 94°C for 2 min and 30 cycles each consisting of 94°C (18 sec), 55°C (21 sec) and 72°C (90 sec). Amplicons were sequenced using primers used for the second step of the semi-nested protocol with the BigDye Terminator reaction kit (Applied Biosystems). Species were determined by phylogenetically comparing sequences with published reference sequences as proposed and provided by Mclntyre *et al.*[19]

Cross-reactivity of EV with HRV was suspected when both EV and HRV PCR were positive and typing resulted in an EV type (9 samples) or when only HRV PCR was positive and typing resulted in an EV type (2 samples). These samples were considered to be EV positive and HRV negative.

Table 1. Genotyping primers used in this study.

Orientation	Name	Sequence
Step 1		
Sense	HRV-VP4-1	GGG ACC AAC TAC TTT GGG TGT
Antisense	9565-reverse	GCA TCI GGY ARY TTC CAC CAC CAN CC
Step 2		
Sense	HRV-VP4-2-forward	GGG GAC CAA CTA CTT TGG GTG TCC GTG T

Bacterial co-infection

At each visit a throat swab was collected which was cultured for respiratory bacterial pathogens according to standard care procedures.

Data analysis

Data were analyzed using SPSS for windows, version 20. Categorical variables were compared by means of chi-square test. Differences between continuous variables were analyzed using student-t test and one way ANOVA test (if normally distributed) or Mann-Whitney U test

and Kruskall-Wallis test and for paired continuous variables Wilcoxon signed rank test. A two-sided p-value <0.05 was considered to be significant.

Results

Subject characteristics

A total of 140 symptomatic and 96 control children were included in the study (Figure 1). Baseline characteristics of all included children are described in table 2. Of the 140 symptomatic children, wheezing was confirmed by the study team in 90 children. Of these wheezing children 74 were visited again by the study team when they were asymptomatic after a minimum of 6 weeks (the recovered group). The median age of the control group (28 months) was significantly higher than of the symptomatic group both during symptoms (15 months, p=0.000) and after recovery (22 months, p=0.000).

Table 2. Characteristics of included children.

	Symptomatic		**Control**	**Recovered**
	RTI with confirmed wheezing	***RTI without wheezing***		
Number of children	90	50	96	74
Median age (months, IQR)	15 (10-25)	15 (10-24)	28 (26-31)[#]	22 (17-27)[#]
Sex (male:female)	1.8:1	1.1:1	1.1:1	1.8:1
Bacterial co-infection	2 (2%)	1 (2%)	2 (2%)	3 (4%)
Use of inhaled corticosteroids	18 (20%)	10 (20%)	-	-
Use of inhaled β2-mimetica	55 (61%)[$]	15 (30%)[$]	-	-
Use of antibiotics	9 (11%)	1 (2%)	-	-
Physician symptom score (median (IQR))	2 (1-4)[$]	0 (0-0)[$]	-	-
mACQ parents (median (IQR))	15.5 (10-21)[$]	11.5 (8-14)[$]	-	-

[#]Significant (p<0.05) for symptomatic versus control and control versus visit after recovery (recovered).
[$]Significant (p<0.05) for RTI with confirmed wheezing versus RTI without wheezing.
RTI; Respiratory tract infection, IQR; Interquartile Range, mACQ; modified Asthma Control Questionnaire.

Prevalence and seasonality of HRV infections

Overall, in 86% of the symptomatic children a respiratory virus could be detected, compared to 40% in the control group (p=0.000) and 53% in the recovered group (p=0.000, Table 3). HRV was the most prevalent virus in symptomatic (41%) as well as control (26%) children, and was found significantly more often in symptomatic children (p=0.009, Figure 2). In the recovered group HBOV (35%) was the most prevalent virus, followed by HRV (25%).

Symptomatic children with HRV infection were significantly younger (median 13.5 months, IQR 8-20) than symptomatic children who were negative for HRV (median 17 months, IQR 13-27, p=0.005). This was also significant in the recovered group (HRV positive; median 19 months, IQR 16-20, versus HRV negative; median 24 months, IQR 18-30, p=0.003).
HRV was seen all year round (Figure 3A) with a peak during fall and winter. In the summer over 80% of symptomatic children were HRV positive contrasted by only 17% during the winter. This was seen in every year of the study. The percentage of asymptomatic children (control and recovered) who were HRV positive was relatively constant between seasons ranging from 17% to 31%.

Table 3. Prevalence of respiratory viruses in symptomatic and asymptomatic (control and recovered) children.

Virus	Symptomatic	Control	Recovered
Any virus	120 (86%)	38 (40%)*	39 (53%)*
Human rhinovirus	58 (41%)	24 (26%)*	19 (25%)*
Human bocavirus	41 (29%)	7 (7%)*	26 (35%)$
Respiratory syncytial virus	33 (24%)	0*	2 (3%)*
Adenovirus	18 (13%)	0*	1 (1%)*
Para-influenzavirus type 3	15 (11%)	1 (1%)*	2 (3%)*
Enterovirus	11 (9%)	3 (3%)	4 (5%)
Human coronavirus	9 (6%)	2 (2%)*	8 (11%)$
Human parechovirus	7 (5%)	0*	3 (4%)$
Humaan metapneumovirus	6 (4%)	1 (1%)	1 (1%)
Para-influenzavirus type 4	5 (4%)	1 (1%)	1 (1%)
Para-influenzavirus type 2	3 (2%)	0	0
Para-influenzavirus type 1	2 (2%)	0	0
Influenza A	2 (1%)	1 (1%)	0
Influenza B	1 (1%)	0	0

*Significant (p<0.05) symptomatic versus control or symptomatic versus visit after recovery (recovered).
$ Significant (p<0.05) control versus visit after recovery (recovered).

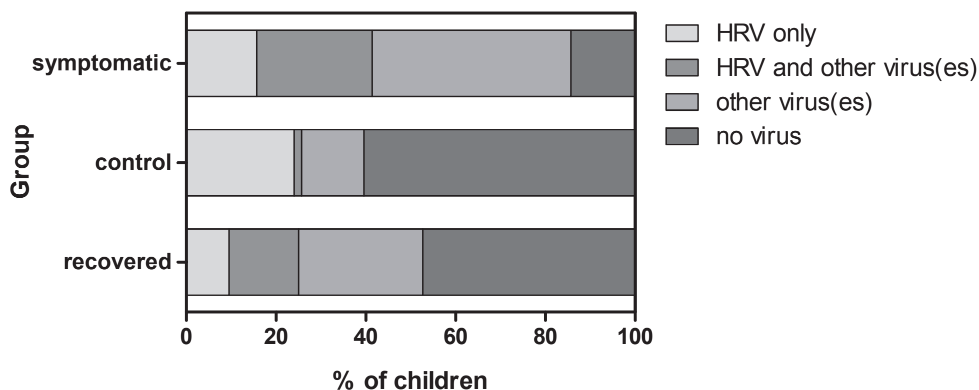


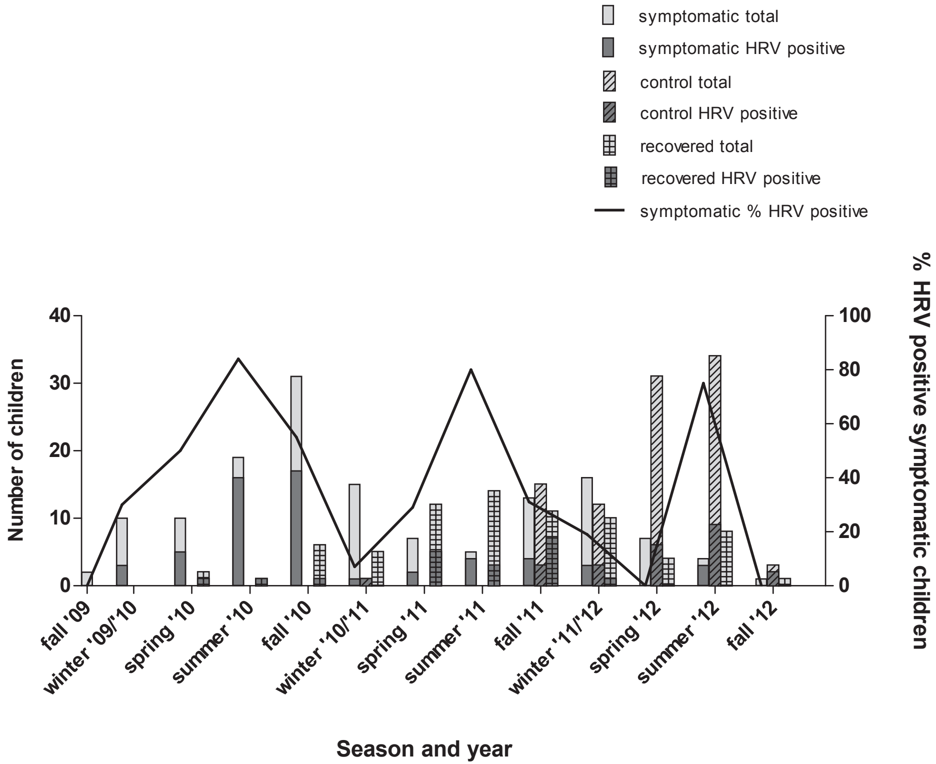
Figure 2. Prevalence of HRV and non-HRV viruses in symptomatic and asymptomatic children (controls and recovered).

Co-infection

In the symptomatic group 44% of the children were infected with 2 or more viruses, significantly more than children in the control group (2%) and recovered children (26%, p=0.000). In addition, the number of recovered children with 2 or more viruses was significant higher than in the control group (p=0.000), which was also reflected by a significantly higher total number of viruses detected (mean number of viruses 0.89) in recovered children compared to children in the control group (mean number of viruses 0.42, p=0.000). This is mainly caused by a higher frequency of HBOV, coronavirus and HPeV (Table 3). Co-infection of HRV with other viruses was found in 36 of 58 (62%) symptomatic children compared to 1 out of 24 controls (4%, p=0.00, Table S1). The most frequent co-infecting viruses were HBOV and adenovirus. There was no significant difference between the co-infection rate in wheezing (55%) and non-wheezing children (75%, p=0.141). For wheezing infants who were recovered from their symptoms, the rate of HRV co-infections was similar to that of symptomatic infants (63%).

The rate of bacterial (co-)infection was low in symptomatic (2%) as well as asymptomatic (control (2%) and recovered (4%)) children (Table 2).

A.



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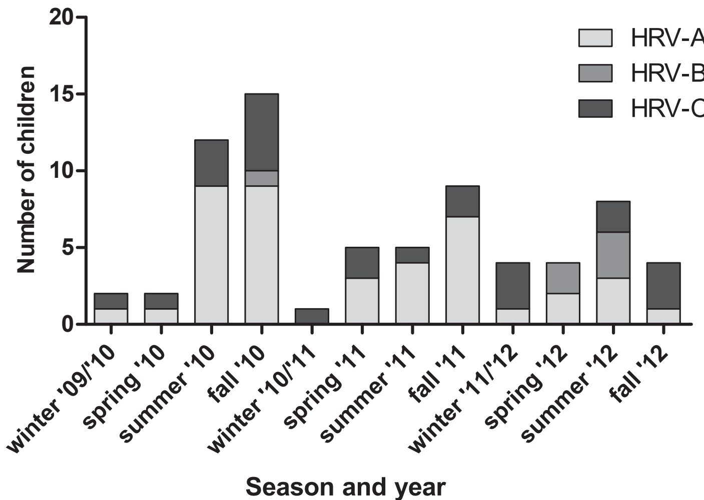

Figure 3. HRV and HRV types in different seasons and years. **A.** Number of HRV positive children in different seasons and years in symptomatic, control and recovered children. **B.** Distribution of HRV-A, -B and –C across seasons and years.

Prevalence of HRV species

In total, 79 samples (71%) could be genotyped (48 samples (76%) of symptomatic children, 19 (70%) of control children and 12 (55%) of recovered children (Table 4). HRV-A was evenly detected across symptomatic (58%), control (50%) and recovered children (78%), while HRV-B was significantly more often detected in control children (31%) compared to symptomatic children (2%, p=0.001). HRV-C was detected in 40% of HRV positive symptomatic children compared to 19% of HRV positive control children and 22% of HRV positive children recovered from symptoms (p=0.25).

In the subgroup of symptomatic wheezing children, children with HRV-C infection were significantly older (median 20 months, IQR 15-27) than wheezing children with HRV-A infection (median 12 months (IQR 8-19, p=0.01).

HRV-A was most frequently seen in the summer and fall, while HRV-C was the most frequently found species in winter. HRV-A, B and C were evenly distributed across the years studied (Figure 3B).

Table 4. Results of molecular typing of HRV PCR positive samples.

Rhinovirus species	HRV-A	HRV-B	HRV-C	Total
Symptomatic	25 (58%)	1 (2%)*	17 (40%)	43
Wheeze	17 (63%)	0	10 (37%)	27
No wheeze	8 (50%)	1 (6%)	7 (44%)	16
Control	8 (50%)	5 (31%)*	3 (19%)	16
Recovered	7 (78%)	0	2 (22%)	9
Total	40 (59%)	6 (9%)	22 (32%)	68

*Significant (p<0.05) symptomatic versus control.
% Indicates percentage of typable HRVs.

Symptoms

HRV and severity of symptoms

Overall, physician symptom scores were low (median 1, IQR 0-3), indicating symptoms were generally mild in this unselected cohort. There were no differences in the scores between HRV positive and HRV negative children. Remarkably, within the group of HRV positive children a significantly higher score was seen in children with HRV mono-infection compared to HRV positive children co-infected with other viruses (median 2 (IQR 0.75-5) versus median 1 (IQR 0-2), p=0.033). The physician symptom scores of children infected with different HRV species were also compared and showed no significant differences.

In addition, the individual symptoms were compared and showed that children with HRV mono-infection and/or HRV-C significantly more often experienced retractions (p=0.016).

Parental assessment of symptoms (mACQ) did not correlate with the physician symptom score and showed a significant lower score in HRV positive children compared to HRV negative children (Table 5, p=0.02), however, the mACQ also assesses general symptoms

of illness (like fever and intake) and upper respiratory tract symptoms while the physician symptom score assesses only severity of symptoms of the lower respiratory tract.

Table 5. Severity scores and symptoms of symptomatic children.

	Total HRV (n=58)	HRV-A (n=25)	HRV-B (n=1)	HRV-C (n=17)	HRV mono-infection (n=22)	non-HRV virus only (n=62)	No virus (n=20)
Physician symptom score (median (IQR))	1 (0-2.25)	1 (0-2)	0	1 (0-2.5)	2 (0.75-5)[1]	1 (0-3)	2 (0-3)
Retractions	19%	8%	0	35%[2]	36%[3]	18%	30%
Use of accessory muscles	17%	16%	0	12%	27%	16%	20%
Wheezing	60%	64%	0	59%	77%	58%	55%
Decreased ventilation	19%	24%	0	12%	18%	19%	10%
mACQ parents (median (IQR))	12 (8.75-16.25)[4]	12 (9-15)	10	12 (7.5-19)	13 (9-22.25)	16 (11-21)	13 (8-16)

[1]Significant higher than HRV positive children with co-infection (p=0.033).
[2]Significant more than HRV-A en HRV-B positive children (p=0.023).
[3]Significant more than HRV positive children with co-infection and HRV negative children (p=0.037).
[4]Significant lower than HRV negative children (p=0.02).

Ct-value and severity of symptoms

Ct value was used as a semi-quantitative read-out for viral load. HRV PCR Ct-values were significantly lower in symptomatic children (throat median Ct-value 30.6, nose median Ct-value 28.6), versus asymptomatic children (throat median Ct-value 31.7 (p=0.009), nose median Ct-value 29.7 (p=0.006)), indicating a higher viral load in symptomatic children. Also, wheezing children had a significant lower Ct-value than non-wheezing symptomatic children (only nose, median Ct-value 27.9 versus 29.1 p=0.024, throat median Ct-value 30.0 versus 30.9, p=0.28, Figure 4). However, a cut-off value for symptomatic disease could not be determined.

There was no correlation between Ct-value and severity of symptoms in HRV infected symptomatic children (Figure 5).

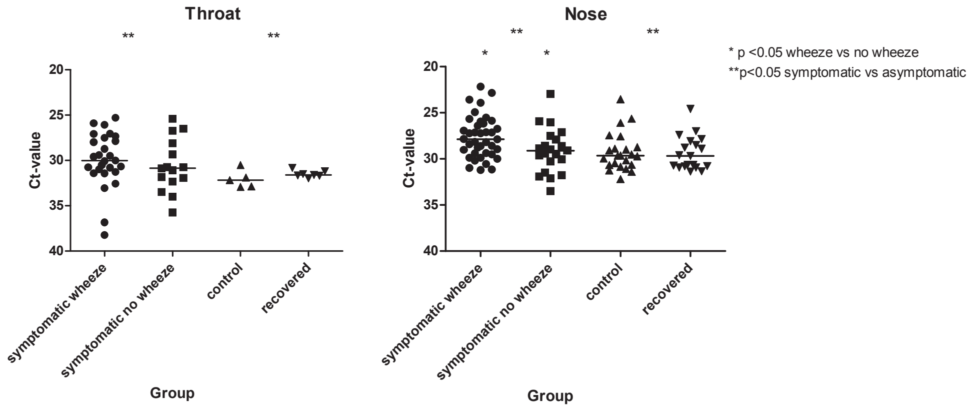

Figure 4. HRV Ct-value in symptomatic (wheezing and non-wheezing), control and recovered children.

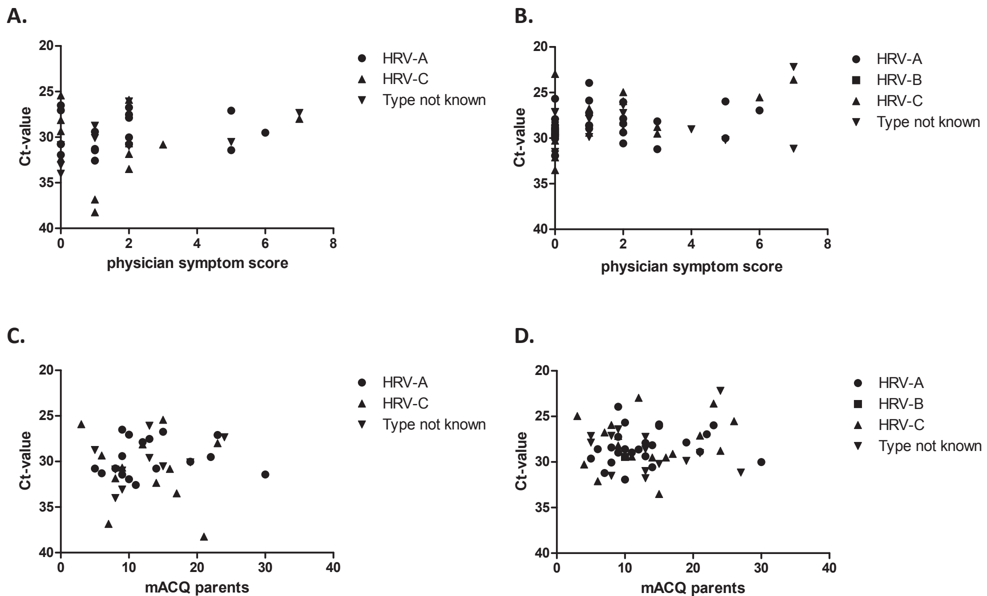

Figure 5. Relation between HRV Ct-value and severity of symptoms. **A.** Throat HRV Ct-value and physician symptom score. **B.** Nose HRV Ct-value and physician symptom score. **C.** Throat HRV Ct-value and mACQ parents. **D.** Nose HRV Ct-value and mACQ parents.

Discussion

Our study is the first to describe the prevalence of HRV in the first years of life in an unselected birth cohort with accurately objectified symptoms and to compare these results with an asymptomatic control group from the same birth cohort.

We showed that HRV infection was very prevalent in symptomatic as well as asymptomatic young children in the general population. HRV was significantly more often detected in

respiratory swabs of symptomatic children compared to asymptomatic children. While HRV mono-infections but not co-infections were associated with a greater clinical severity, HRV infections were not associated with occurrence of wheezing. However, wheezing HRV infected children had a significantly higher HRV viral load than non-wheezing HRV infected children. In addition, there was no association between the different HRV species and occurrence of wheezing or severity of disease. In this unselected birth cohort no association between HRV-C and wheezing or more severe disease was found.

HRV-A and C were found to be the most prevalent species detected in our unselected population. This is in accordance with previous reports in both hospitalized patients and high-risk cohorts [11,13,22-30] as well as unselected birth cohorts.[31,32]
HRV-B was the least prevalent species and seemed to be associated with asymptomatic infection in our study. This is in accordance with Lee *et al.*[22] who showed that HRV-B is less virulent than HRV-A and HRV-C in an high risk cohort of young children. However, others found that HRV-B was associated with pneumonia in hospitalized children.[23]
The lack of association between wheezing or more severe disease and HRV-C infections in this study is in contrast with several other studies who reported an association between HRV-C and more severe disease and/or more asthma exacerbations in hospitalized patients.[11,13,14,27] However, this was not consistently reproduced.[15,23,28]
Studies in unselected cohorts or non-hospitalized symptomatic children also showed different results, ranging from no association of wheezing in children infected with HRV-C,[31,33] to more (severe) lower respiratory tract infections in children infected with HRV-C.[32]
These conflicting results indicate a discrepancy between various studies with respect to associations of HRV-C infection with wheezing and severity of symptoms. It could be possible that specific HRV types of the HRV species C (or A) give more severe disease, including wheezing illness and hospitalization, as was suggested by Lee *et al.*[22] Another explanation could be that this association is only seen in susceptible children above a certain age. This is supported by our observation and from others that wheezing children with HRV-C infection were significantly older than HRV-A infected wheezing children.[32]

Our study showed that a higher viral load was associated with symptomatic disease, and within the symptomatic group, with wheezing. Unfortunately, a cut off value of viral load for symptomatic disease could not be established due to considerable overlap between groups. We also did not find a correlation between severity of disease and Ct-value. Few other studies described the relation between viral load and symptoms. Kennedy *et al.*[34] found no difference in viral load between outpatient wheezing and non-wheezing children, while others found that a high viral load was associated with symptomatic disease and an increased risk of LRTI, but not with more severe symptoms.[17,28,35]
An explicit strength of our study is that children were visited and sampled both during the symptomatic period and after recovery from symptoms. Remarkably, a significantly higher

number of (co-infecting) viruses was found after recovery from symptoms compared to controls. It is possible that wheezing children are more prone to be (asymptomatic) carriers of viruses, compared to non-wheezing children. However, during the symptomatic visit no difference between wheezing and non-wheezing children and number of viruses detected was seen. In our study, control children were significantly older than recovered children, making it impossible to draw conclusions from this observation alone. In addition, in another large cross-sectional study in asymptomatic children a consistent high viral detection rate of 58-74% in 6, 12, 18 as well as 24-month old children was found with HRV as the most prevalent virus (31-50%) and multiple viruses in 18-38% of the children.[36] No data on preceding wheezing illness were available.

This brings us to the first limitation of this study; the children in the control group were significantly older than symptomatic infants, thereby biasing the comparison of prevalence between symptomatic and asymptomatic children. This is an unfortunate secondary effect of the fact we chose to study asymptomatic controls that never met the criteria for a symptomatic episode from the same birth cohort. This means we would have required to recruit a novel control when a former control became symptomatic. We do however feel this is also an implicit strength as it means our control group is very strictly defined. In addition, it is important to notice that comparisons of severity of symptoms and wheezing between HRV species within the symptomatic group were not influenced by this bias.

Furthermore, 44% of the symptomatic infants were infected by multiple viruses. Although this is in accordance with previous studies, it is likely to have influence on the analysis of associations between HRV species and clinical severity.[13,23,30,37,38] Remarkably, we found that children with a HRV mono-infection had more severe symptoms compared to children with HRV and a co-infection. Other hospital-based studies found that co-infection with RSV is associated with an increased severity and/or a longer duration of hospitalization.[13,38] In our cohort co-infection with RSV was low and not associated with more severe symptoms.

Another limitation is the sample size. It is possible that the lack of association of HRV-C with more severe symptoms or wheezing is due to an insufficient sample size. We evaluated this with a post hoc power analysis, showing that our study had enough power to pick up large differences in wheezing frequency or symptom severity, however, small differences could have been missed (data not shown).

In conclusion, in an unselected birth cohort from the Netherlands with mild respiratory disease, we found a high prevalence of (multiple) respiratory viruses with HRV being the most prevalent. We found no association between HRV infection and wheezing illness, although a higher HRV viral load was seen in wheezing children. We did find that children infected with only HRV had more severe disease than HRV negative children and children with HRV and co-infections. We did not establish a correlation between HRV-C infection and wheezing or more severe disease, suggesting that HRV-C is not associated with more severe disease in young children in the general population.

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Table S1. Frequency of HRV co-infections with different viruses.

	Symptomatic			Control	Recovered
	Total	***Wheeze***	***No wheeze***		
Any virus	36 (62%)	21 (55%)	15 (75%)	1 (4%)*	12 (63%)$^{\$}$
Human bocavirus	21 (36%)	15 (40%)	6 (30%)	1 (4%)*	11 (58%)$^{\$}$
Adenovirus	11 (19%)	8 (21%)	3 (15%)	0*	0*
Enterovirus	6 (10%)	3 (8%)	3 (15%)	0	1 (5%)
Human parechovirus	6 (10%)	4 (11%)	2 (10%)	0	1 (5%)
Respiratory syncytial virus	5 (9%)	4 (11%)	1 (5%)	0	1 (5%)
Para-influenzavirus type 4	4 (7%)	2 (5%)	2 (10%)	0	0
Para-influenzavirus type 3	3 (5%)	3 (8%)	0	0	1 (5%)
Human coronavirus	2 (3%)	1 (3%)	1 (5%)	0	1 (5%)
Para-influenzavirus type 2	1 (2%)	1 (3%)	0	0	0
Human metapneumovirus	0	0	0	0	1 (5%)

*Significant ($p<0.05$) symptomatic versus control and/or symptomatic versus visit after recovery (recovered).
$^{\$}$ Significant ($p<0.05$) control versus visit after recovery (recovered).

Chapter 3

Clinical relevance of positive human parechovirus type 1 and 3 PCR in stool samples

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Abstract

Human parechoviruses (HPeV) cause symptoms ranging from severe neonatal infections to mild gastrointestinal and respiratory disease. Use of PCR and genotyping has markedly improved the detection rate of HPeV but has simultaneously raised questions about the clinical relevance of positive tests. This retrospective study correlates positive HPeV1 or HPeV3 PCR tests in stools from children with their symptoms to determine clinical relevance.

Children with HPeV1- or HPeV3-positive stool samples, as detected by real time RT-PCR and direct genotyping, between 2004 and 2008 were selected. Clinical data were retrospectively collected from the patient's files and results were compared.

One hundred and thirty-eight children with positive HPeV1 (n=112) or HPeV3 (n=26) stool samples were identified. Significantly more HPeV3-infected children were neonates or infants younger than 6 months of age. Meningitis or sepsis-like illnesses were diagnosed most frequently and were found in significantly younger children. Almost half of HPeV1-infected children had an underlying disease. Mild gastrointestinal disease was seen most frequently in these children.

There was no clear correlation between viral load (Ct value) and severity of symptoms.

In conclusion, HPeV3 detected by PCR in stool samples is associated with clinically relevant disease. For HPeV1, a positive stool sample is mainly associated with symptoms in children with underlying disease.

Introduction

Human Parechovirus (HPeV) is increasingly recognized as an important cause of sepsis-like illness (SLI) and meningitis in young children.[1,2] There are 16 known HPeV types, of which HPeV1 and HPeV3 are the most prevalent.[3,4] Like enteroviruses, HPeV can cause a wide range of symptoms and disease from mild gastrointestinal or respiratory disease to SLI and meningitis. HPeV1 and -2 usually evoke mild gastrointestinal and respiratory symptoms, but occasionally HPeV1 infection can elicit severe disease, such as encephalitis, transient paralysis and myocarditis in children.[5-7]

Human parechovirus type 3 was first detected in 2004 in Japan [8] and is associated with neonatal SLI and meningitis in several studies.[9-11]

The recently identified HPeV4, -5 and -6 [12-14] seem to be associated with mild respiratory and gastrointestinal symptoms,[15] but the number of described cases is still low. Clinical data on HPeV7-16 infections are rare or lacking.

Previously, we and others showed that HPeV3 infection is associated with SLI and meningitis and that HPeV3-infected children are significantly younger than HPeV1-infected children.[4,9] The number of patients in these initial studies was small and virus was detected by conventional cell culture from stool. The clinical relevance of presence of HPeV3 in cerebrospinal fluid (CSF) was clearly demonstrated.[2,10,16]

The use of PCR and direct genotyping has increased the detection frequency of HPeV in stool samples;[3,17,18] however, the clinical relevance of PCR detection in stool samples is still unknown. Although some show associations between HPeV1 presence in stool samples and gastrointestinal symptoms,[18] others suggest that HPeV positivity in stool has little clinical importance and is an innocent bystander.[19]

In this retrospective study we describe the demographic, clinical and microbiological characteristics of a large group of children with HPeV1 and HPeV3 infections. Correlations between positive PCR findings and clinical symptoms and between viral load in stool and severity of disease are determined.

Materials and Methods

Detection of HPeV-positive patients by real-time RT-PCR and genotyping

At the Laboratory of Clinical Virology of the Academic Medical Center (AMC) in Amsterdam, the Netherlands, stool samples that had been routinely stored at -80°C between January 2004 and December 2006 were retrospectively screened for HPeV using an HPeV-specific real time RT-PCR,[3,17] using a standardized amount of stool. Stool samples obtained from 2007 onward were prospectively screened as part of routine diagnostics by the real-time RT-PCR. All stool samples were tested for suspected viral infections (enterovirus and/or HPeV) as indicated by the treating paediatrician, even in the absence of gastrointestinal symptoms.

The Ct value of the real-time RT-PCR was used as a semi-quantitative read-out. A Ct-value >40 was considered negative.[3,20,21] Direct genotyping was performed on all HPeV-positive stool samples by sequencing the complete VP1 region, as described by Benschop *et al.*[3,17]
For this study, children with HPeV1- and HPeV3-positive stool samples who visited the Academic Medical Center (a tertiary academic hospital) or the Amstelland Hospital Amstelveen (a general regional hospital) between January 2004 and December 2008 were selected. The Academic Medical Center is a referral hospital for children with complex diseases, and it also serves as a general hospital for their region.

Clinical data

Data on clinical signs and symptoms of children infected with HPeV were retrospectively collected from the patient files. The child's age at time of HPeV detection, gender and duration of hospital stay were documented. An infection was labelled as hospital-acquired when the HPeV-positive stool sample was obtained >6 days after hospital admission. Medical records and letters of discharge at time of HPeV detection in stool samples were used to collect data on the presence and duration of fever (temperature >38.5°C), SLI (signs of circulatory and/ or respiratory dysfunction defined by tachycardia or bradycardia, low blood pressure and/ or decreased saturation), neurological symptoms (irritability, meningitis (defined as >19 leukocytes/µL in CSF for children aged 0-28 days and >9 leukocytes/µL for children aged 29 days and older [22] with or without elevated protein level (>0.35 g/L) and/or decreases in glucose level (<2.8 mM) and/or positive HPeV PCR), encephalitis, seizures or paralysis), gastrointestinal symptoms (diarrhoea, nausea and/or vomiting), respiratory symptoms (rhinorrhoea, cough, otitis, tonsillitis, signs of dyspnoea, tachypnoea, wheezing, inspiratory stridor and abnormalities on chest radiography), skin symptoms, use of antibiotics, and diagnosis at discharge. If a specific symptom was not clearly mentioned in the medical record or letter of discharge, the symptom information was labelled as 'missing'.
Available laboratory results of blood (leukocyte count and C-reactive protein) and CSF analysis (cell count, protein and glucose level), results of neuro-imaging (cranial ultrasound, computed tomography, magnetic resonance imaging) and the presence and site of isolation of other micro-organisms were also recorded. A final diagnosis was made for every child based on available clinical data. Patients were divided into five categories: gastroenteritis, severe infection, respiratory tract infection, infection not otherwise specified and other. Infection not otherwise specified was defined as a suspected viral illness with or without fever and no clear localizing symptoms and reported as such by the treating paediatrician. Other was used to categorize all non-infectious diagnoses.

Statistical analysis

Data were analysed using SPSS for Windows, version 19/20. Clinical symptoms were compared using a chi square test. Differences between means were compared using Student's *t* test and one way analysis of variance or Kruskall-Wallis and Mann-Whitney *U* test. A p value <0.05 was considered to be significant.

Results

Patient characteristics

During the study period of 5 years, 138 children with positive HPeV1 or HPeV3 stool samples were identified. HPeV1 was found in 112 patients and HPeV3 was found in 26 patients (Table 1). One patient was infected with both types subsequently (HPeV3 at the age of 3 weeks and HPeV1 at the age of 2.5 months).

In both groups, boys were more frequently infected with either HPeV1 or HPeV3 than girls (Table 1, HPeV1 59%, HPeV3 65%).

Significantly more HPeV3-infected children were <6 months at the time of diagnosis (p <0.05), with a median age of 2 months at the time of HPeV3 diagnosis compared with 6 months for children with an HPeV1 infection. Of the HPeV3-infected children, 15.4% were neonates (younger than 28 days), whereas only 2.7% of the HPeV1-infected children were younger than 28 days (p <0.05).

Almost half of the children (45%) with HPeV1 infection had an underlying disease compared with only 12% of HPeV3-infected children (p <0,05). Most children had cardiorespiratory disease (21%), followed by gastrointestinal disease (15%) and neurological disease (13%).

Hospital-acquired infections were not significantly more often found in children with HPeV1 infections. The proportion of children that were not admitted to the hospital was 19% in HPeV1-infected children and 28% in HPeV3-infected children. However, children with an HPeV1 infection were admitted for longer than HPeV3-infected children (median 9 versus 5 days).

Other pathogens were found in 37% of the HPeV1-positive children and in 20% of the HPeV3-positive children (Table 1). In the HPeV3 group, co-infection with enterovirus was seen in the majority of patients. Viruses were also the most frequently detected co-pathogens in the HPeV1 group, especially rotavirus and enterovirus.

More than half (52%) of the children infected with HPeV3 received antibiotics whereas one-third of HPeV1-infected children received antibiotics (p=0.07).

Infections with HPeV1 were found all year round, whereas HPeV3 was detected exclusively in summer and autumn. HPeV3 was found biannually in the years 2004, 2006 and 2008, whereas HPeV1 was detected annually (Fig. 1).

Table 1. Characteristics of children with positive stool samples for human parechovirus type 1 (HPeV1) or type 3 (HPeV3).

		HPeV with and without co-infection		HPeV without co-infection	
Characteristic		**HPeV1**	**HPeV3**	**HPeV1**	**HPeV3**
Number of children (%)		112 (81)	26 (19)	71 (78)	20 (22)
Academic Medical Center (%)		85 (85)	15 (15)	57 (84)	11 (16)
Amstelland Hospital (%)		27 (71)	11 (29)	14 (61)	9 (39)
Gender: Male (%)		66 (59)	17 (65)	39 (55)	13 (65)
Age at time of diagnosis (months)					
Median (range)		6 (0-66)	2 (0-30)	6 (0-54)	2 (0-30)
<6 months (%)		48 (43)	18 (69)*	34 (48)	14 (70)
Neonate (%)		3 (3)	4 (15)*	1 (1)	4 (20)*
Underlying disease (%)[a]		50/112 (45)	3/25 (12)*	35/71 (49)	2/20 (10)*
Prematurity (%)[a]		14/111 (13)	4/25 (16)	9/70 (13)	2/20 (10)
Presentation with suspected infection (%)[a]		71/109 (65)	21/25 (84)	41/70 (59)	16/20 (80)
Hospital-acquired infection (%)[a]		23/112 (21)	3/25 (12)	15/71 (21)	3/20 (15)
Not admitted (%)[a]		21/111 (19)	7/25 (28)	17/71 (24)	5/20 (25)
Duration of hospitalization (days)					
Median (range)		9 (1-276)	5 (2-80)	9 (1-276)	5 (2-80)
Antibiotics given (%)[a]		35/107 (33)	13/25 (52)	19/68 (28)	9/20 (45)
Other microorganism detected (%)[a]		41/112 (37)	5/25 (20)		
Faeces	Enterovirus	11	4		
	Rotavirus	15	0		
	Adenovirus	1	0		
	Bacteria	4	0		
	Parasite	0	1		
Blood	Bacteria	4	0		
	Parasite (malaria)	1	0		
Cerebrospinal fluid	Bacteria	2	0		
Nasopharyngeal aspirate	Enterovirus	1	0		
	Other virus	7	0		
Sputum	Bacteria	2	0		
Abdominal drain fluid	Bacteria	1	0		

[a]Data are displayed as number of children with characteristic/number of children of which presence or absence of characteristic is known.
*p <0.05 for comparison between HPeV1- and HPeV3-positive children.

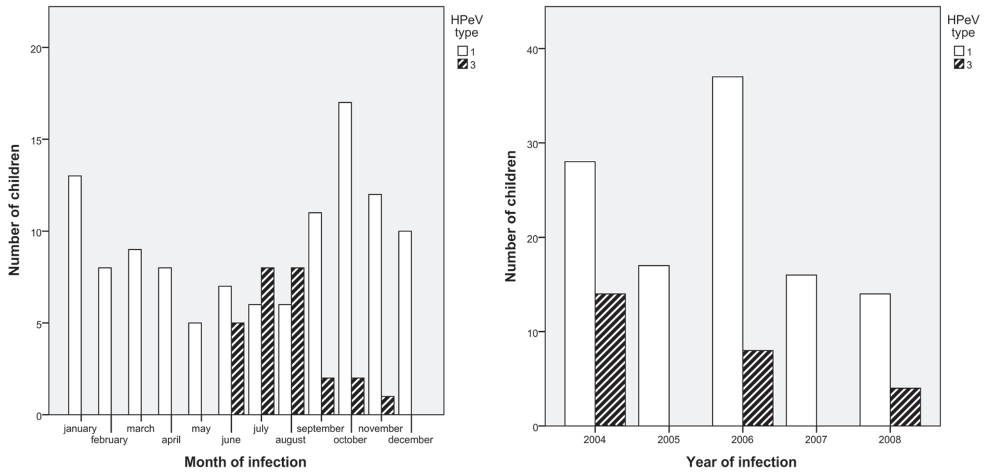

Figure 1. Prevalence of human parechovirus type 1 (HPeV1) and type 3 (HPeV3) in various months and years.

Clinical characteristics; HPeV3 is associated with more severe disease

Clinical data were available for the majority of the children (Table 2).

Gastrointestinal symptoms were most frequently reported and were found more often in children with HPeV1 infection than in HPeV3-infected children (95% versus 77%, p <0.05). Fever was recorded equally in both groups. Significantly more children with an HPeV3 infection had neurological symptoms (33 versus 8%, p <0.05) or sepsis-like illness (24 versus 5%, p <0.05) as compared with HPeV1-infected children. HPeV PCR was performed on blood in only nine children with HPeV1 (four HPeV PCR-positive) and in two children with HPeV3-positive stool samples (both HPeV PCR-positive).

The HPeV PCR was performed on CSF samples of 13 children (five HPeV3 and eight HPeV1) with a clinical suspicion of meningitis. None of the eight HPeV1-positive children had a positive HPeV PCR in CSF whereas all five children with HPeV3 had a positive HPeV PCR in CSF. Other pathogens were found in the CSF of two of the HPeV1-positive children (*Streptococcus pneumoniae* in a previously healthy child and a coagulase-negative *Staphylococcus* in a child with a ventriculo-peritoneal drain).

In HPeV1-positive children an elevated white blood cell count was recorded in 67% of nine CSF specimens available for cell count. None of the children with HPeV3 had an elevated CSF white blood cell count (of three CSF specimens available for cell count).

Skin symptoms were significantly more common in HPeV3-infected children compared with HPeV1-infected children (38 versus 9%, p <0.05). Exanthema was most frequently described in HPeV3-positive children.

Table 2. Clinical symptoms of children with human parechovirus type 1 (HPeV1) and type 3 (HPeV3) infection.

	No. of patients with HPeV1 and HPeV3 infection with and without other co-infections (%)			No. of patients with HPeV1 and HPeV3 infection without other co-infections (%)		
Clinical symptoms	**HPeV1**	**HPeV3**	**Total**	**HPeV1**	**HPeV3**	**Total**
Fever	47/106 (44)	12/24 (50)	59/130 (45)	23/67 (34)	10/19 (53)	33/86 (38)
Sepsis-like illness	5/107 (5)	6/25 (24)*	11/132 (8)	1/68 (1)	5/20 (25)*	6/108 (6)
Neurological symptoms	8/104 (8)	8/24 (33)*	16/128 (13)	4/67 (6)	6/19 (32)*	10/86 (12)
Meningitis[a]	6/8	5/5		3/6	3/3	
Irritability	6/8	7/8		3/4	5/6	
Convulsions	2/8	0/8		2/4	0/6	
Gastrointestinal symptoms	101/106 (95)	20/26 (77)*	121/132 (92)	65/67 (97)	16/20 (80)*	81/87 (93)
Diarrhoea	89/99	17/21		56/64	14/17	
Blood in stools	12/88	1/20		7/56	1/16	
Nausea/vomiting	52/97	4/20		27/62	3/16	
Skin symptoms	9/104 (9)	9/24 (38)*	18/128 (14)	6/66 (9)	7/19 (37)*	13/85 (15)
Respiratory symptoms	42/103 (41)	3/24 (13)*	45/127 (35)	25/65 (39)	2/19 (11)*	27/84 (32)
Upper respiratory tract symptoms	33/42	3/3		20/25	2/2	
Otitis	14/38	0/3		9/24	0/2	
Lower respiratory tract symptoms	14/42	1/3		6/25	1/2	

Data are displayed as number of children with documented symptom/number of children with documentation of presence or absence of characteristic.
[a] Defined as elevated white blood cell count in cerebrospinal fluid (CSF) and/or HPeV PCR-positive in CSF.
*p <0.05 for comparison between HPeV1- and HPeV3-positive children.

Respiratory symptoms were seen more frequently in children with HPeV1 (41%) versus HPeV3-infected children (13%, p <0.05), and were mainly described as upper respiratory tract symptoms. The HPeV PCR was performed on nasopharyngeal aspirate in ten children with HPeV1-positive stools (five HPeV PCR positive) and none of the children with HPeV3-positive stools.

Overall, children with HPeV3 infection had significantly more severe disease (SLI and/or meningitis) as the final diagnosis in comparison with children with HPeV1 infection (35% versus 3%, p <0.05, Table 3).
For children with an HPeV1 infection, gastroenteritis was the most common final diagnosis (71%). Most of these children had mild gastroenteritis but were admitted for other reasons or were diagnosed with HPeV1 while hospitalized for other reasons. Severe gastroenteritis, leading to hospitalization was only described in 17% of the children with detection of HPeV1 as a sole pathogen (Table 3).

To determine in what proportion of HPeV PCR-positive stool samples symptoms were attributable to HPeV infection, children with a non-infectious final diagnosis (defined as 'other' in Table 3) or the presence of another pathogen were excluded. This resulted in 51% of the HPeV1-positive children and 72% of the HPeV3-positive children with an infection as final diagnosis in whom HPeV was the only pathogen found.
In the group of HPeV3-infected children, the mean age of children with severe disease (3.7 months; SD ± 8.4 months) or with an infection not otherwise specified (2.4 months; SD ± 3.2 months) was significantly lower than children with gastroenteritis (16 months; SD ± 11.8 months). In the HPeV1 group there were no age-related differences in diagnosis.

Table 3. Final diagnosis of children with human parechovirus type 1 (HPeV1) and type 3 (HPeV3) infection.

	No. of patients with:			
Final diagnosis	**HPeV1 without and with co-infections (%)**	**HPeV1 without co-infections (%)**	**HPeV3 without and with co-infections (%)**	**HPeV3 without co-infections (%)**
Gastroenteritis	75 (71)	47 (72)	6 (24)*	4 (20)*
Other reason for admittance, during stay gastroenteritis	34 (32)	25 (39)	3 (12)	3 (15)
Gastroenteritis mild (no admittance)	13 (12)	11 (17)	3 (12)	1 (5)
Gastroenteritis severe (admittance)	28 (27)	11 (17)	0	0
Severe infection	8 (8)	2 (3)	9 (36)*	7 (35)*
SLI	3 (3)	2 (3)	4 (16)	4 (20)
Meningitis	0	0	2 (8)	1 (5)
SLI and meningitis	0	0	3 (12)	2 (10)
Bacterial sepsis and/or meningitis	4 (4)	0	0	0
Pericarditis	1 (1)	0	0	0
Respiratory tract infection	5 (5)	1 (2)	0	0
Upper respiratory tract infection	2 (2)	1 (2)		
Lower respiratory tract infection	3 (3)	0		
Infection NOS	4 (4)	4 (6)	8 (32)*	7 (35)*
Other	13 (12)	11 (17)	2 (8)	2 (10)
Total	105 (100)	65 (100)	25 (100)	20 (100)

SLI, sepsis-like illness; infection NOS, infection not otherwise specified.
*p <0.05 for comparison between HPeV1- and HPeV3-positive children without and with co-infection and for comparison between HPeV1- and HPeV3-positive children without co-infection.

No clear correlation between Ct value in stool and severity of disease

To investigate whether viral load detected in stool by PCR was correlated with severity of clinical symptoms, the Ct value of the real-time 5' untranslated region PCR was used as a semi-quantitative read-out.[20,21] The overall mean Ct value of the stool samples was

low (indicating high viral loads) in both HPeV1 (26.2 ± 4.9 SD) and HPeV3 (26.7 ± 4.1 SD) -infected children (Fig. 2).
Stool samples with high Ct values are often difficult to culture [17] and are therefore considered of less clinical relevance. Benschop *et al.*[17] found that the mean Ct value, at which HPeV culture became negative but PCR remained positive in faeces, was 35.
None of the HPeV3-infected children had a Ct value >35, whereas eight of the HPeV1-infected children had Ct values of >35. In the HPeV1-infected children there was no association of Ct value with either final diagnosis, presence of co-infection, underlying disease or severity of symptoms.

Remarkably, in the group of HPeV3-infected children without co-infection, Ct values were significantly higher (mean Ct value of 31.1 ± 1.2 SD, range 30.1-33.0) in children presenting with severe infection (SLI and/or meningitis), compared with children with other diagnoses (mean Ct value of 25.1 ± 3.8 SD, range 18.7-32.3).

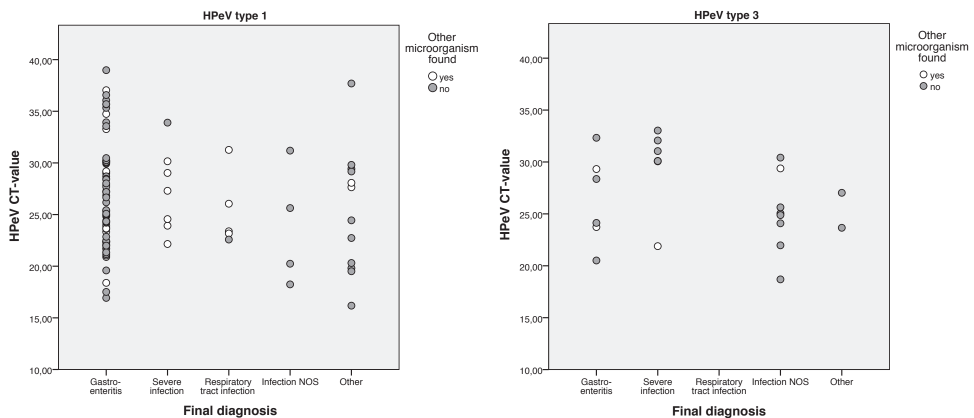

Figure 2. Ct-value in faeces and final diagnosis of children infected with human parechovirus type 1 (HPeV1) and type 3 (HPeV3).

Discussion

In this study, we describe a comprehensive clinical analysis of HPeV1-infected and HPeV3-infected children diagnosed by real-time RT-PCR and direct genotyping of stool samples, focusing on the relevance of PCR as a diagnostic method for HPeV infection in stool samples. A positive HPeV3 stool sample is, in the vast majority of cases, associated with relevant clinical disease. HPeV1-positive stool samples are mainly of clinical relevance in children with underlying disease.

In this large patient group diagnosed by PCR we confirm our previous results that infection with HPeV3 is significantly more associated with severe disease in younger children compared with infection with HPeV1.[9] Although those results were based on virus detection by virus culture from stool in a small group of patients, they were confirmed by other studies from Europe and North America.[2,4,10,16,23-25] Our results also show that HPeV3-infected children with severe disease are significantly younger than HPeV3-infected children with gastroenteritis. Earlier studie*s* described that more serious disease is usually seen in younger children,[9-11,23] but this study is the first to show a significant difference.

In a study in Japanese children with HPeV infection detected by PCR in stool samples, low numbers of severe disease in both HPeV1- and HPeV3-infected children were found.[18] The age of the Japanese HPeV3-infected children was markedly older (average 12.1 months) than the HPeV3-infected children in this study. This is in agreement with our observation that severe disease is seen in younger children.

Seroprevalence studies showed that 92-99% of adults have antibodies against HPeV1.[26] In children, seroprevalence of HPeV1 is around 30% at the age of one year, increasing to >70% at the age of two years,[27] indicative of a high infection rate of HPeV1 in young children. Seroprevalence of HPeV3 is low (<2.7%) in children and does not increase much in adulthood (10-13%) in Europe.[26] The absence of neutralizing HPeV3 antibodies in adults could be an explanation for the occurrence of HPeV3 infection at very young ages with more severe symptoms, as young children are not protected by maternal antibodies.

The role of HPeV1 in causing clinical symptoms and disease is yet to be determined. Although some studies [19,28,29] report lack of associations between clinical symptoms and HPeV1 detection, other studies [30] found associations between HPeV1 infection and otitis media. As in earlier studies [4,24] we found that HPeV1 infection is mainly associated with (mild) gastroenteritis and detected mostly in children with an underlying illness or in combination with other pathogens, which corroborates that HPeV1 is of low clinical importance and so can be seen as an innocent bystander in certain cases, as suggested by Zhang *et al.*[19]

In accordance with earlier reports from the Netherlands, we confirm that HPeV1 is seen year round, with peaks in the winter season, whereas HPeV3 is mainly seen in summer and autumn.[4,9] Earlier studies from our centre showed that in 2004-2006 HPeV PCR was positive in 16.3% of stool samples of children under the age of 5 years, with HPeV1 and HPeV3 being the most predominant types.[3] In 2007-2008 HPeV PCR was positive in 8.6% of stool samples, detected mainly in children under the age of 5 years.[17]

In contrast with HPeV1, for which prevalence was relatively stable over the years, HPeV3 was almost exclusively found in even calendar years as described before in the Netherlands [3,4,23] and the UK.[2] Other studies in Europe [16,25] and Japan [8,18] did not confirm this bi-annual circulation. Interestingly, in the USA HPeV3 is detected mainly in the summer of uneven calendar years.[10,11] The underlying reason for these differences remains unknown.

Conclusion

In a large group of HPeV-infected children we show that HPeV3 detected by PCR in stool samples is associated with younger age and more severe disease (SLI and/or meningitis) and is clinically relevant when detected, regardless of Ct value.
HPeV1 is predominantly found in susceptible patients like those with underlying disease or in combination with other infectious pathogens. HPeV1 mainly elicits (mild) gastrointestinal symptoms, and its detection in stool is of less clinical relevance than HPeV3 detection.

Acknowledgements

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3

Chapter 4

Prolonged shedding of human parechovirus in feces of young children after symptomatic infection

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Both authors contributed equally to this manuscript

Submitted as brief report

Abstract

After symptomatic human parechovirus (HPeV) infection in infants, the duration of (mostly asymptomatic) shedding in feces was 2-24 weeks (median 58 days). HPeV Ct-value could neither differentiate between symptomatic disease and asymptomatic shedding nor between severe and mild disease as high Ct-values (indicating low viral loads) were observed in HPeV3 infected children with severe disease.

Introduction

Human parechoviruses (HPeV) are small non-enveloped, positive-sense, single-stranded RNA viruses and belong to the family of *Picornaviridae*. Today, 16 types are known of which HPeV type 1 and 3 are the most prevalent. HPeVs are closely related to human enteroviruses (EVs), giving similar symptoms ranging from mild gastrointestinal and respiratory disease to life-threatening infections like meningitis/encephalitis and sepsis-like illness (SLI) (reviewed in Harvala *et al.*[1]).

In recent years molecular techniques became state-of-the-art in clinical diagnostic settings; HPeV specific real-time PCR was designed to detect HPeV in all kinds of patient materials.[2,3] Like EVs, the main site of replication of HPeV is the gastrointestinal tract, and HPeV PCR from fecal samples is very sensitive for the diagnosis of HPeV infection, even in the absence of gastrointestinal symptoms.[4] Longitudinal studies in cohorts of young children showed that both EV and HPeV can be detected for several months in feces of children.[5-7] The clinical relevance and relation with symptoms of a positive PCR in feces for both EV and HPeV is yet unclear.

In this study the duration and extent of HPeV shedding in feces of infants after symptomatic HPeV infection is described. In addition, we compared the amount of virus detected in feces in children with severe and mild disease.

4

Methods

Subjects

This study is part of the PARMA (PARechovirus infections and Maternal Antibodies) study, a prospective multicenter case-control study to evaluate presence of maternal antibodies in young children with HPeV infection. Children under the age of 1 year with a proven HPeV infection and their mothers were defined as cases and were selected for this study.

Data on clinical signs and symptoms of included children were collected from the patient's files and discharge letters. A final diagnosis was made for every child based on available clinical data. Patients were divided in severe and mild disease. Mild disease was defined as gastroenteritis and/or respiratory tract infection, or non-localized symptoms of a viral infection without any signs of SLI. Severe disease was defined as meningitis/encephalitis and/or myocarditis/pericarditis and/or SLI. SLI was defined as signs of circulatory and/or respiratory dysfunction defined by tachycardia or bradycardia, low blood pressure and/or decreased saturation.

Duration of shedding

The parents of children with a positive HPeV PCR in feces were asked to collect a feces sample every 2 weeks until HPeV PCR in feces was negative. Clinical symptoms at the moment of sample collection were documented during telephone contacts and recorded.

Virus detection and genotyping

HPeV and EV real-time reverse transcription (RT-)PCR was performed on available samples (feces, CSF, blood, nasopharyngeal aspirate) as described earlier.[8] The cycle threshold (Ct)-value was used as a semi-quantitative read-out.[8] A Ct-value of 40 or more was considered negative.[3] HPeV positive feces samples were genotyped by sequencing the complete VP1 region as described previously.[8]

Results

Patient characteristics

In total 38 HPeV infected children were included in the study between 2008 and 2012. The median age was 2 months, ranging from 5 to 352 days. More boys than girls were included (ratio 1.5:1).

HPeV types

Feces samples were collected within one week after the start of symptoms. HPeV3 was the most frequently detected type (n=22, 58%), followed by HPeV1 (n=8, 21%) and HPeV4 (n=6, 16%). HPeV6 was detected in one child. In one child HPeV typing was not successful.
Co-infection with other microorganisms was detected in 40% of the children at time of first sampling. Another virus was found in samples of 12 children of which EV was the most frequent co-infecting virus (7 feces samples). Bacterial co-infection was found in 4 samples. Significantly more HPeV1 and HPeV4 infected children had a co-infection with another microorganism (respectively 75% and 83%) compared to HPeV3 (18%, p=0,000).

Duration of HPeV shedding

Of the parents of the 38 HPeV positive children, 30 agreed to collect feces every two weeks until HPeV PCR became negative. The parents of three children discontinued collecting follow-up feces. Of the remaining 27 children, 7 were positive for HPeV1, 16 for HPeV3, 3 for HPeV4 and 1 for HPeV6. The initial Ct-value in feces of these children varied between 16 and 30 (Fig. 1). The median duration until HPeV RNA became undetectable was 58 days (range 2-24 weeks). In general, the viral load decreased gradually over time (Fig. 1).
A rise in viral load (a decrease in Ct-value of more than 5 between 2 time points) was seen in five patients. Two patients (P3-6 and P6-1, Fig. 1) acquired a new HPeV1 infection (one had diarrhea and the other was without symptoms). One patient (P1-3) experienced a novel

episode of diarrhea and was co-infected with adenovirus. One patient (P4-4) was co-infected with EV from week two onwards and did not have any symptoms. One patient (P1-5) was still infected with the same HPeV type and did not have any symptoms.

Of the remaining patients, three elicited clinical symptoms after the initial HPeV diagnosis. One patient (P1-2) was admitted 3.5 weeks after initial HPeV diagnosis with diarrhea caused by an adenovirus infection. Two other patients (P3-13 and P1-7) experienced an episode of diarrhea respectively 2 and 9 weeks after initial HPeV diagnosis. PCR was negative for other gastrointestinal associated viruses in these patients.

There were no significant differences in duration of shedding between HPeV1, -3 and -4 positive children. There was no relation between age and duration of shedding nor between severity of disease and duration of shedding.

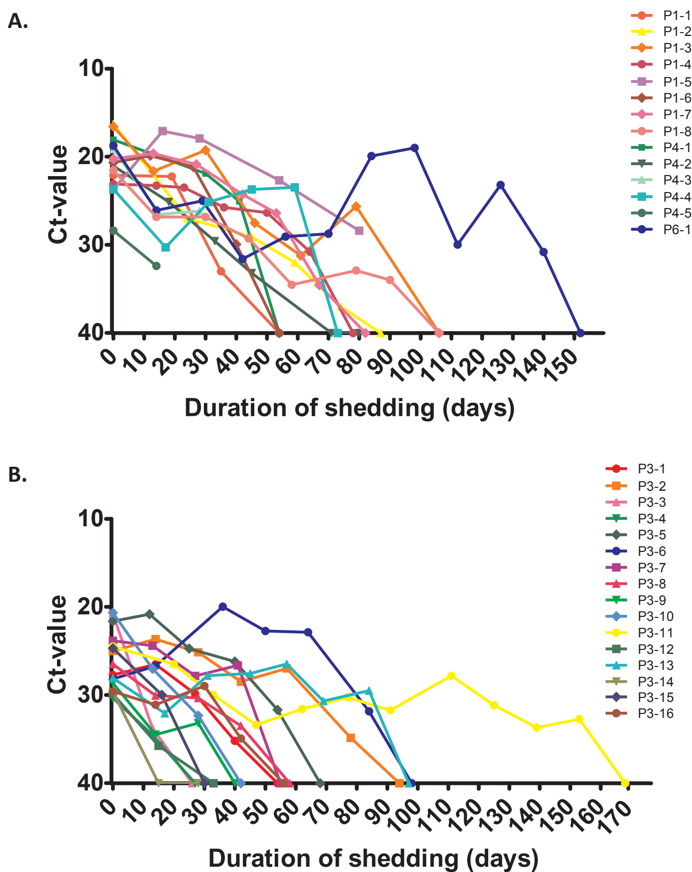

Figure 1. Duration of shedding and Ct-value in feces of HPeV1, HPeV4 and HPeV6 infected children **(A)** and HPeV3 infected children **(B)**.

Disease severity and Ct-value

At diagnosis, the mean Ct-value in feces of HPeV3 infected children (mean 26.9 ± SD 3.8) was significantly higher than the mean Ct-value of HPeV1 infected children (mean 20.5 ± SD 2.8, p=0.000). Although significantly more HPeV3 infected children had severe disease (11/22 patients) compared to HPeV1 (2/8 patients) and HPeV4 (0/6 patients) infected children (p=0.03), the initial Ct-value in feces did not differ significantly between children with severe disease and mild disease in total and per HPeV type (Fig. 2). However, Ct-values of 30-35 were seen in two of 10 (20%) HPeV3 infected children with severe disease.

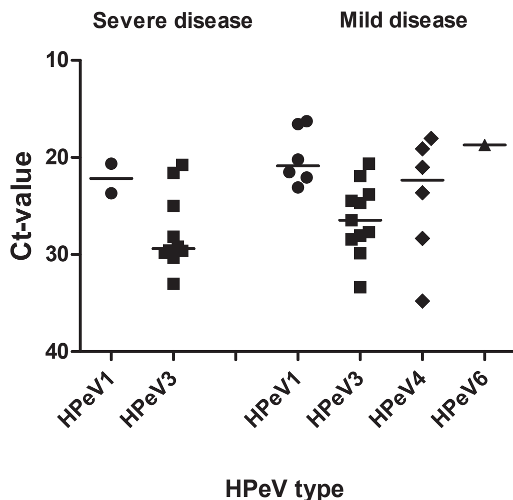

Figure 2. Initial Ct-value in feces of symptomatic HPeV infected children.

Discussion

In infants with symptomatic HPeV1, -3 and -4 infection, asymptomatic HPeV shedding in feces occurred for a prolonged period of time (up to 6 months) after initial infection. Most children had no (gastrointestinal) symptoms during the period of shedding. Ct-value (as semi-quantitative measurement of viral load) could not differentiate between symptomatic disease and post-infectious shedding, although in the majority of children a trend towards higher Ct-values/lower viral loads was seen over time.

Longitudinal studies (of several years) in Norway [5] and Finland [7] described detection of HPeV in stool samples up to 5 months in healthy children above the age of 3 months. In those studies mainly HPeV1 was reported and there was no association between occurrence of HPeV shedding and clinical symptoms.

In our study the majority of the children was younger than 3 months and infected with HPeV3, showing that post-infectious shedding also occurs in very young infants with HPeV3 infection.

The longevity of asymptomatic shedding of HPeV in feces makes it difficult to interpret a positive HPeV PCR result from feces, because a relation with clinical symptoms is not always present. Especially in HPeV1 and -4 infected children, a high rate of co-infection with other microorganisms, mainly viruses, was found. Thus, a positive HPeV PCR in feces must be interpreted with caution, taking into account other possible causative organisms and diseases. However, we and others showed that HPeV1 and especially HPeV3 are able to cause (severe) disease [4,9] and that HPeV is not only an innocent bystander as was suggested in a recent study.[10]

We found no significant difference in HPeV Ct-value in feces between symptomatic infection and asymptomatic post-infectious shedding. This confirms the results of earlier studies, in which high viral loads were found in asymptomatic children.[10,11]

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In addition, the initial Ct-value during symptomatic disease did not differ between children with mild and severe disease, but Ct-value in HPeV3 infected children was significantly higher as compared to HPeV1 and -4 infected children. In a previous study we found that the Ct-value in children with severe HPeV3 disease (mainly meningitis/encephalitis) was significantly higher than in HPeV3 infected children with other diagnoses.[4] The lower viral load in feces can be a result of differences in cell tropism of HPeV3 as is suggested by Westerhuis *et al.*[12]; HPeV3 strains which caused CNS symptoms in the patients they were derived from, showed better replication kinetics in neural cell lines. In addition, replication kinetics on one gastrointestinal cell line (Caco-2) were high for all HPeV3 strains, but low or absent on another gastrointestinal cell line (HT29) while replication for HPeV1 was high on both cell lines. These results have to be interpreted with caution since they are derived from *in vitro* experiments with continuous growing cell lines, which do not necessarily represent gastrointestinal cells *in vivo*. However, our results implicate that even high Ct-values in feces of HPeV3 infected children can be of clinical relevance and secondly that viral load in feces is not a good instrument for evaluating severity of infection.

In conclusion: After symptomatic infection, shedding of HPeV1, -3 and -4 in feces can occur for months in infants. Viral load (Ct-value) cannot differentiate between asymptomatic shedding and symptomatic infection nor between severe and mild disease.

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Chapter 5

Exposure to recreational and drinking water and the occurrence of parechovirus and enterovirus infections in infants

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**** Both authors contributed equally to this manuscript***

Submitted as brief report

Abstract

In this case-control study among hospitalized young children, human enterovirus and parechovirus infection status of patients and controls were related to questionnaire data. We showed that drinking water consumption and recreational water exposure were no significant risk factors for acquiring infection. However, familial spread was potentially associated with virus infection.

Introduction

Human enterovirus (EV) and parechovirus (HPeV) infections often remain asymptomatic but can also result in symptomatic diseases in young children. EV and HPeV can elicit a variety of diseases; while Coxsackie A viruses and HPeV1 mainly cause rashes and mild gastrointestinal disease, polioviruses and enterovirus 71 can cause severe neurological disease, and HPeV3 infections lead to severe and life-threatening complications in infants.[1]
The transmission route of many members of the family of *Picornaviridae*, such as EV and HPeV, is mainly fecal-oral, by ingestion of contaminated food or water or through person-to-person contact. However, the specific sources for virus exposure have not been clarified, hampering targeted interventions. Few reported outbreaks have been attributed to drinking water and recreational water exposure,[2] and person-to-person spread was shown in two cases of sepsis-like illness caused by HPeV3 which could be traced back to their older siblings.[3] Furthermore, secondary person-to-person spread may cloud the initial infection source such as ingestion of contaminated water, which may go unnoticed.
Surface water can be contaminated with EVs and HPeVs by several sources. High numbers of these viruses are discharged with treated and untreated sewage water,[4] which are thus an abundant source of contamination. Although the number of infectious viruses in the environment will gradually decrease over time due to inactivation and removal, enteric viruses are known to persist for several weeks up to months.[5] The high prevalence of these viruses in environmental waters may pose a health risk to people that come into contact with contaminated water. Especially when vulnerable individuals, such as infants and young children, are exposed to these contaminated aquatic environments, exposure may pose a public health threat. The goal of this study was to test the hypothesis that exposure to water (drinking water and recreational water) is related to diseases caused by EV and HPeV infections in infants admitted to the hospital, and their families.

Study design

The case-control study PARMA (PARechovirus infection and Maternal Antibodies) aimed to determine if maternal antibodies protect children against HPeV infection, as described elsewhere.[6] The current study was conducted among children younger than 1 year included in the PARMA study; those admitted to the hospital with a confirmed EV or HPeV infection as cases and those matched by age and similar clinical symptoms to the cases (with negative EV and HPeV PCR results) and were admitted to the hospital for other medical reasons (2009-2012) as controls. Questionnaires about the consumption of water and contact with water through recreational activities were given to the parents at the time of inclusion or were sent retrospectively to part of the participants of the PARMA-study after their consent. Data on the consumption of drinking water by the mothers as well as by the children through

consumption of prepared milk powder, and recreation in diverse waters by the whole family (swimming pool, surface water, seawater) were collected. The questions referred to the three months prior to the first clinical manifestations displayed by the patient. When answers could not be remembered exactly, approximate answers were to be supplied, noting this at the end of the questionnaire. A physician helped the mothers of the newly included children to fill out the questionnaires. The association between exposure factors and virus infection (EV and HPeV) were examined univariably with logistic regression.[7] Those variables displaying a *p*-value <0.30 were analyzed multivariably. Given the relatively low number of cases, variables with a *p*-value <0.10 were considered statistically significant and values between 0.10 and 0.20 as potentially associated and of interest for further studies. All statistical analyses were performed using SPSS 19 software package (SPSS Inc., Chicago, IL, USA).

Results

Approximately 63% (54/85) of the water related questionnaires were returned. Of the responders 24 questionnaires were from cases with a confirmed HPeV infection, 12 were from cases with a confirmed EV infection and the remaining 18 were from hospitalized control subjects. Of the 24 confirmed HPeV infections in the infants, 13 were typed as an HPeV3, six were HPeV1, three were HPeV4, one was HPeV6 and one remained untyped. In the feces of 12 infants with an EV infection included in this study 11 different EV types were detected, showing the diversity of EVs circulating in the human population causing serious infections in young children. Several echoviruses, Coxsackie A and B viruses were found. The average age of HPeV3 infected infants was much younger than infected children with an HPeV infection other than type 3, and with an EV infection; 42 days old, 111 days old and 95 days old, respectively.

The majority of the families (15 out of 16) who had reported that they had swum in the 3 months prior to the disease development of the infant visited a swimming pool while only one family had been swimming in surface water; swimming in seawater was not reported (Table 1). The newborn of the family that had been swimming in surface water developed an echovirus 11 infection. Of the 54 mothers in this study, 32 were breastfeeding their child, 14 of those also gave powdered milk to their child and 22 were solely formula fed. For the preparation of the powdered milk, unboiled water (8), boiled water (16), both unboiled and boiled water (2), and mineral water (2) were used. Different volumes were used in the preparation of powdered milk but most parents used 120-180 mL per feeding, in case of additional formula feeding (besides breastfeeding) water volumes were significantly lower (30-50 mL).

No risk factors for acquiring an EV or HPeV infection were statistically significantly associated with the consumption of unboiled tap water or water recreation by infants (Table 1). A

potential association between the infected infants and the presence of other children in their family was observed, most frequently one (22), but also two (10), three (1) and even four siblings (1) were present.

Table 1. Characteristics of the children included in the study and the summary of possible risk factors, percentage found in cases with a confirmed virus infection, their odds ratio (OR), 95% confidence interval (CI), and the p-value. Totals not adding to 54 per factor are due to missing data.

Factor			Total	% Cases	OR	95% CI	P-value
Sex child:	male		33	63.6	1.0		
	female		21	71.4	1.4	0.44 – 4.7	0.552
Age child:	<90 days		34	67.6	1.0		
	>90 days		20	65.0	0.89	0.28 – 2.9	0.842
Siblings		no	20	55.0	1.0		
		yes	34	73.5	2.3	0.71 – 7.3	0.167
Child, during week:	home (100%)		37	67.6	1.0		
	daycare/family (part)		13	69.2	1.1	0.28 – 4.2	0.515
	hospital (100%)		3	33.3	0.24	0.02 – 2.9	0.489
Health complaints family		no	31	61.3	1.0		
		yes	17	76.5	2.1	0.54 – 7.8	0.279
Swimming pool:	mother	no	44	68.2	1.0		
		yes	9	55.6	0.58	0.14 – 2.5	0.473
	baby	no	49	67.3	1.0		
		yes	4	50.0	0.48	0.62 – 3.8	0.492
	family	no	38	66.8	1.0		
		yes	15	66.7	1.0	0.29 – 3.7	0.952
Food child:	breastfeeding	no	22	68.2	1.0		
		yes	32	65.6	0.89	0.28 – 2.8	0.845
	formula feeding	no	16	62.5	1.0		
		yes	36	66.7	1.2	0.35 – 4.1	0.771
Powdered milk	unboiled water	no	41	68.3	1.0		
Preparation:		yes	10	60.0	0.70	0.17 – 2.9	0.619
	boiled water	no	30	56.6	1.0		
		yes	18	72.2	1.9	0.56 – 7.0	0.276
Drink mother:	unboiled water	no	4	75.0	1.0		
		yes	50	66.0	0.65	0.062 – 6.7	0.707
	boiled water	no	32	65.6	1.0		
		yes	37	40.5	1.1	0.35 – 3.6	0.845
	mineral water	no	40	75.0	1.0		
		yes	10	50.0	0.43	0.11 – 1.8	0.244

Discussion

In this study, we could not identify risk factors associated with exposure of infants to water for acquiring human EV or HPeV infections in infants. Although the response was relatively high, the resulting sample size was relatively small. This limitation may have reduced the power of the risk factor analyses. Nevertheless, the included children resulted in a representative group of HPeV, EV and control individuals.

The lack of identification of water-associated risk factors for young children in acquiring an EV or HPeV infection in this Dutch cohort may be indicative for a high quality of tap water and recreational waters in the Netherlands. This is not unexpected, because the microbial safety of the drinking water is safeguarded by a preventative quantitative risk assessment approach as laid down in the Dutch drinking water directive.[8] With respect to Dutch recreational water quality the EU bathing water directive is leading which has now been implemented in Dutch legislation.[9] No information was derived on possible person-to-person transmission, because this study did not focus on other factors e.g. hygiene. Furthermore, mother and infant contact and the presence of maternal antibodies was not included in the present study.

Infections with EV or HPeV can be mild or even asymptomatic, and these asymptomatic infections are probably a continuing source infecting other susceptible individuals, in case of infected children in infecting their sibling(s). Particularly very young children are at risk for development of severe disease after an EV or HPeV infection, most likely because of their often immature immune system.

In a previous study, HPeV1 was detected as the most prevalent type in sewage (20/89 samples), while HPeV3 was detected less frequently (8/89) in the period 2010-2011 in the Netherlands,[10] reflecting the HPeV types that were circulating in the human population at that time. The higher percentage of HPeV3 infections determined in this hospital-based study was not surprising, because the clinical manifestations of HPeV3 infections are generally more severe as compared to other HPeV infections.

Notably, the age of the infants with an HPeV3 infection was lower than the age of the children with an EV infection. Children with an HPeV3 infection were mainly determined in the even years (12/13). The biannual occurrence and the young age of HPeV3 infected children have been described extensively.[1,11]

The high diversity of circulating EVs in the children was also observed in our screening of environmental samples in 2010-2011 (unpublished data). Schets *et al.*[12] reported that in the Netherlands, swimming in surface water mainly occurs in the summer months (July-August) of the bathing season, whereas the children included in the PARMA-study were included in a period of four years. The three months prior to the time of infection in many cases did not overlap with these two summer months, possibly explaining the low reporting of swimming in surface waters.

In conclusion, although we could not identify significant risk factors for acquiring EV or HPeV infections associated with exposure to water in our study, a potential association between the infected infants and the presence of other children in their family was suggestive of spread of the viruses from their siblings to the infant.

Acknowledgements

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PART II

Treatment options

Chapter 6

The need for treatment against human parechoviruses: how, why, and when?

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Abstract

Since 1999, human parechoviruses (HPeVs) have been classified as a separate group in the large and expanding family of *Picornaviridae*. In contrast to the well-established group of the human enteroviruses (HEVs), HPeVs have long been considered as irrelevant and have only been associated with mild disease manifestations in children. The identification of HPeV-3 in 2004 and its association with neonatal sepsis, refocused attention on this neglected group of viruses. Clinically HPeV infections may mimic HEV infections and are increasingly recognized as viral causes of sepsis-like illness and CNS infections in young children. Therapy is not available against HPeVs or HEVs. In this article, we will demonstrate that therapy against this group of picornaviruses is urgently needed and we will review the current knowledge of treatment options as well as the current developments in antiviral therapy against picornaviruses in the scope of treatment possibilities against HPeVs.

Introduction

Human parechoviruses (HPeVs) show many resemblances to human enteroviruses (HEVs) with respect to genome structure, cytopathological effect (CPE) in cell culture, and clinical manifestations.[1-3] Detection of HPeV-1 and -2, previously known as echovirus-22 and -23, used to be part of enterovirus diagnostics by virus culture, showing an indistinguishable CPE on the same cell lines. While the structure of the genomes of HPeV and HEV are very similar, the nucleotide sequences of the HPeVs are relatively distinct from the HEVs. Therefore, separate molecular techniques are necessary to detect HPeVs. The development of molecular methods has led to a rapid expansion of the group of HPeV that now contains 14 genotypes.[4] By comparison, the HEV group contains over 100 serotypes. Infections with HEV are very common and the clinical course is usually mild. Nevertheless, HEVs are also the major viral cause of CNS infections (e.g. meningitis, encephalitis and acute flaccid paralysis), as well as neonatal sepsis and myocarditis.[5,6] Neonatal sepsis caused by HEVs can be fatal, [5,7-9] which is also illustrated by a recent alert for increased severe neonatal sepsis caused by coxsackievirus B1 in the USA.[10] Encephalitis by HEV is a rare condition but sequelae are reported at a high frequency in these patients.[11] In patients with a humoral immunodeficiency, HEV infections can manifest as chronic meningoencephalitis with continuing detectable HEV in cerebrospinal fluid (CSF) and ongoing clinical symptoms.[6] These clinically severe conditions warrant therapy to stop continuing viral replication, and possibly decrease disease burden and prevent complications. Despite substantial effort to develop safe and effective antiviral drugs against HEVs, there is currently no therapy available.

Similar to infections with HEV, infections with HPeV are very common and the clinical course is usually mild, but occasionally they are associated with neonatal sepsis-like illnesses and possibly even with sudden infant death.[12-15] In addition, as HPeVs have also been identified as a significant cause of viral CNS infections that may lead to severe sequelae,[14,16,17] effective therapy against HPeV infections is imperative.

HPeV biology

Classification & biology

Human parechoviruses (HPeVs) are single-stranded, positive-sense RNA viruses within the *Parechovirus* genus of the large *Picornaviridae* family. The *Picornaviridae* family currently consists of 13 genera: *Enterovirus*, *Parechovirus*, *Hepatovirus*, *Cosavirus*, *Kobuvirus*, *Aphthovirus*, *Erbovirus*, *Teschovirus*, *Cardiovirus*, *Tremovirus*, *Sapelovirus, Avihepatovirus* and *Senecavirus.* Furthermore, two new genera, *Klassevirus* and *Aquamovirus,* have recently been proposed (Figure 1A). The genus *Enterovirus* contains over 200 different virus types known to infect humans, include rhinoviruses (HRVs), echoviruses, coxsackie-A and -B viruses (CAV and CBV), polioviruses and numerically identified enterovirus 68-109. By

contrast, all human hepatitis A viruses in the genus *Hepatovirus* belong to a single serotype and are responsible for acute hepatitis. The genus *Aphthovirus* includes seven foot-and-mouth disease virus (FMDV) serotypes, which are very important pathogens of cloven-hoofed animals worldwide.

The first two serologically distinct HPeV types were discovered over 50 years ago during a summer diarrhea outbreak in the USA.[18] These prototype strains were originally described as echovirus-22 and -23 in the *Enterovirus* genus; the clinical presentation - enterovirus-like CPE on virus isolation and non-pathogenicity in both mice and monkeys - led to their original designation as enteric cytopathic human orphan (echo)viruses. However, they were renamed as HPeVs and reclassified into their own genus in 1999 based on evident differences in genome organisation and structure, divergence of encoded proteins and other biological properties.[1,19] Since this reclassification, a further 12 HPeV types (HPeV-3 to -14) have been identified (reviewed in Harvala *et al.*[20]; Table 1). In addition, a close relative of HPeV, Ljungan virus (LV), has been classified as a separate parechovirus species. LV has been primarily isolated from rodents (Figure 1B).[21]

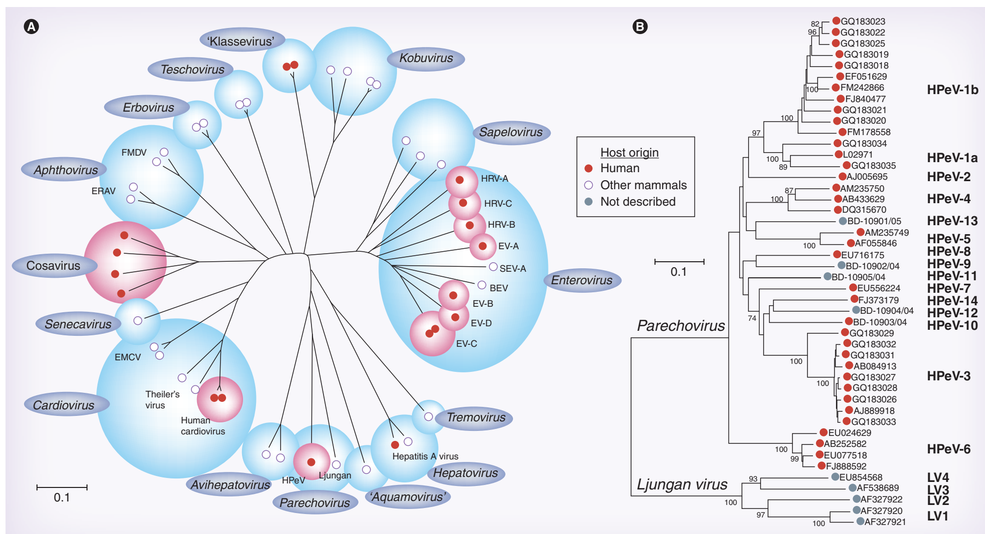

Figure 1. Evolutionary relationship of human parechovirus to other picornaviruses: classification into genera and species. **(A)** Evolutionary relationship of HPeV to other picornaviruses: classification into genera and species. Evolutionary tree of picornaviruses showing its division into 13 designated and two proposed genera ('*Klassevirus*' and '*Aquamovirus*'). The tree was constructed by comparisons of amino acid sequences of the 3Dpol region of representative viruses from each designated genus and species (positions 5711–7252 in the prototype Harris genome [accession number L02971]). **(B)** Genetic heterogeneity within the *Parechovirus* genus: classification into two species. Comparison of nucleotide sequences from VP1 region (position 2336-3028) of all currently classified 14 HPeV types and four Ljungan virus types. Type designations are shown on the right.

BEV: bovine enterovirus; EMCV: encephalomyocarditis virus; ERAV: equine rhinitis virus; EV: enterovirus; FMDV: foot and mouth disease virus; HPeV: human parechovirus; HRV: human rhinovirus; LV: Ljungan virus; SEV: simian enterovirus.

Data from [4,201].

Table 1. Human parechovirus genotype reference strains and clinical association.

Genotype	Strain	Accession	Clinical association	Study (year)	Ref.
HPeV-1(A)	Harris	S45208	GIT and RT symptoms, bronchiolitis, pneumonitis, otitis media, encephalitis†, paralysis†, myocarditis†	Hyypia *et al.* (1992)	19
HPeV-1(B)	BNI788st	EF051629		de Souza Luna *et al.* (2008)	98
HPeV-2	Williamson	AB084913, AJ889918	GIT and RT symptoms	Ghazi *et al.* (1998)	99
HPeV-3	A308/99, Can82853-01	AB084913, AJ889918	Neonatal sepsis, meningitis, encephalitis and paralysis†	Ito *et al.* (2004) Boivin *et al.* (2005)	39 13
HPeV-4	K251176-02, T75-4077	DQ315670, AM235750	Fever and GIT and RT symptoms	Benschop *et al.* (2006) Al-Sunaidi *et al.* (2006)	100 101
HPeV-5	CT86-6760, T92-15	AF055846, AM235749	Fever, GIT and RT symptoms, Sepsis† and Reye's syndrome†	Oberste *et al.* (1998) Al-Sunaidi *et al.* (2006)	102 101
HPeV-6	NII561-2000	AB252582	Fever, GIT and RT symptoms, paralysis† and Reye's syndrome†	Watanabe *et al.* (2007)	47
HPeV-7	PAK5045	EU556224		Li *et al.* (2009)	60
HPeV-8	BR/217/2012	EU716175	Enteritis	Drexler *et al.* (2009)	59
HPeV-9	BAN2004-10902			Oberste MS	unpublished data
HPeV-10	LK-106/LK-103	GQ402515/ GQ402516	Gastroenteritis	Pham *et al.* (2010)	58
HPeV-11	BAN2004-10905			Oberste MS	unpublished data
HPeV-12	BAN2004-10904			Oberste MS	unpublished data
HPeV-13	BAN2004-10901			Oberste MS	unpublished data
HPeV-14	451564	FJ373179		Benschop *et al.* (2008)	35

†Sporadically reported.
GIT: Gastrointestinal tract; HPeV: Human parechovirus; RT: Respiratory tract.

The HPeV genome is approximately 7300 bases in length, and encodes a single polyprotein flanked by 5' and 3' untranslated regions (UTRs; Figure 2A). RNA released into the cell on virus entry is directly translated into a long polyprotein, which is subsequently cleaved by the viral protease (3C) into three structural proteins (VP0, VP1 and VP3) and seven nonstructural proteins (2A-2C and 3A-3D). The RNA-dependent RNA polymerase (3D) copies genomic RNA to make a template from which genomic and mRNA transcripts can be generated. In addition to a role in formation of membrane-associated replication complexes,[22] 2C protein shows NTPase activity and binds RNA.[23] Without direct observational data, it can only be inferred from comparison with better characterised picornaviruses that 3B (VPg) is likely to be attached to the 5' end of the genomic RNA and has a functional role in initiation of HPeV transcription. In most picornaviruses, there are four structural proteins (VP1-4) that form the virus nucleocapsid (Figure 2B), but the maturation cleavage of VP0 into VP2 and VP4 does not appear in parechoviruses (Figure 2A).[24] However, the external appearance of HPeV

particles has been recently shown by cryoelectron microscopy and image reconstruction and has proved consistent with the external appearance of other picornaviruses, most closely resembling FMDV in the *Aphthovirus* genus.[25] The mechanism by which the large VP0 protein is released or externalized during the life cycle of HPeV is currently unknown. Also, in contrast to other picornaviruses, the predominant antigenic sites of HPeV have been mapped to the N-terminal region of the VP0 protein, in a region that is not found to be antigenic in any other genera.[26] Antiserum against a synthetic peptide representing this region showed neutralizing activity. In addition, peptide antiserum against the C-terminal region of HPeV-1 VP1 protein, which contains the arginine-glycine-aspartic acid (RGD) motif, was also neutralizing. Neutralizing antibodies are thought to be critical in the control of picornavirus infections: it was demonstrated a long time ago that paralytic poliomyelitis follows the viremia and that neutralizing antibodies can prevent a poliomyelitis disease.[27] However, no data on whether neutralizing antibodies are elicited during HPeV infections, or their role in preventing disease and conferring immunity, have been obtained.
Human parechovirus replication is dependent on sequence elements and RNA secondary structures at the 5'UTR (and likely 3'UTR) of the genome. Specifically, a long hairpin stem-loop and pseudoknot interaction with a downstream RNA structure in the 5'UTR have been shown to be required for HPeV replication.[28] Much of the rest of the 5'UTR forms a complex RNA structure with a demonstrated role as an internal ribosomal entry site (IRES).[29] This directs ribosomal binding to a position close to an internal methionine codon (position 710 in the HPeV-1 Harris strain) from which translation commences. IRES-mediated translation is found in all picornaviruses, although the structure of the IRES varies considerable between genera. The HPeV IRES is classified as type 2, structurally similar to those found in *Aphthovirus*, *Cardiovirus* and *Erbovirus* genera.[30]

Human parechoviruses differ from most other picornaviruses by not shutting off host cell protein synthesis during replication. In HEVs this is principally achieved by cleavage of the elF-4G subunit of the cap-binding complex by the 2A protein. This provides many picornaviruses with a replication advantage in preventing normal cap-dependent translation of cellular RNA, while enabling IRES-dependent translation to proceed. However, the parechovirus 2A protein is unlikely to possess a protease activity; indeed, its binding to the 3' end of the HPeV genome suggest its vital role in virus replication.[2]

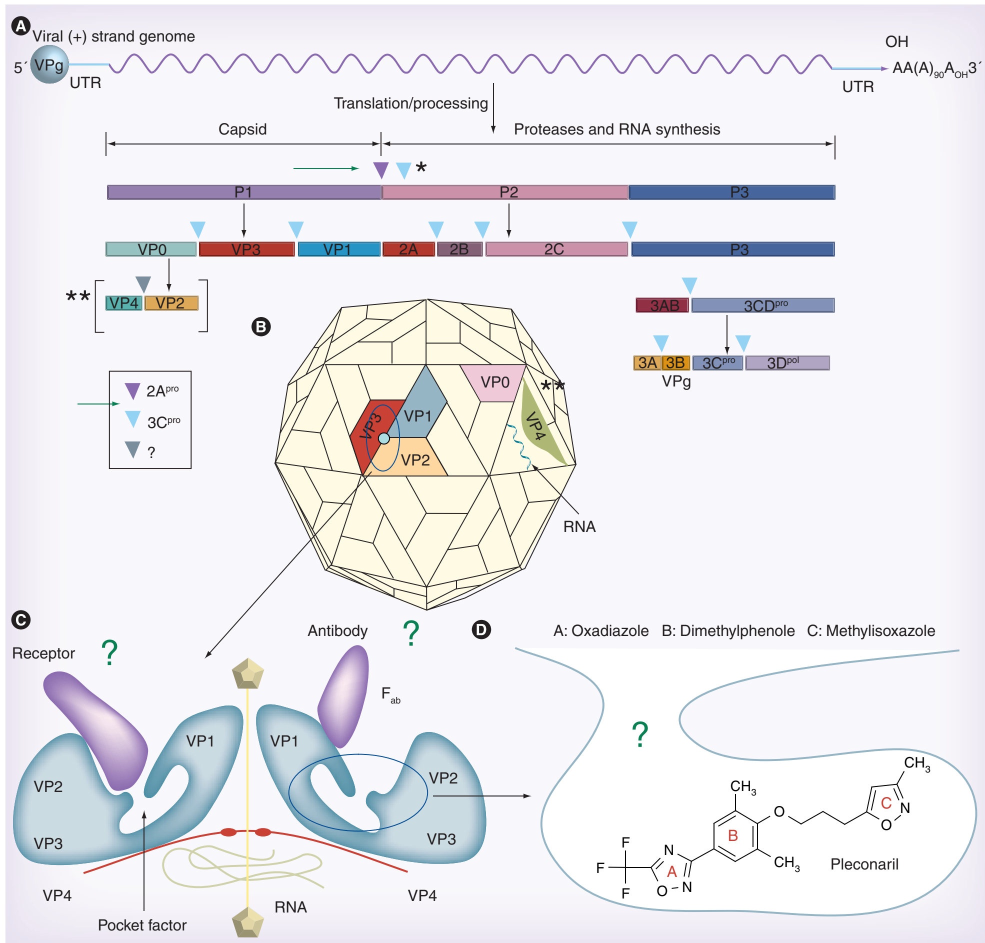

Figure 2. Capsid structure and genome organization of the human parechovirus and human enterovirus genomes with the main targets for antiviral therapy. **(A)** The viral genome, with the genome-linked protein VPg at the 5'-end, the 5'UTR, an open reading frame of approximately 7 kB, the 3'UTR, and the poly(A) tail. The coding region is divided into three regions: P1, the structural region encoding the capsid; P2 and P3, the non-structural region encoding proteinases and polymerases. The large polyprotein is cleaved by viral proteinases. In human parechoviruses (HPeVs) only one protease, 3Cpro(*****), is involved in processing. In human enteroviruses (HEVs) and human rhinovirus (HRV), P1 is cleaved from P2 by 2Apro, and an as yet unknown protease is responsible for cleavage of VP0 (in parenthesis, ******). In HPeV, cleavage of VP0 does not occur. The green arrow indicates the two proteinases as the target for inhibition by protease inhibitors. **(B, C & D)** Viral capsid and the hydrophobic pocket. The capsid **(B)** consists of 60 protomers formed by the capsid proteins VP1 (blue), VP3 (red) and VP0 ** (pink) for HPeV, and VP2 (orange) and VP4 (green) for HEV and HRV. At the junctions of the capsid proteins lies a canyon with a hydrophobic pocket, important for receptor binding. **(C)** model of HEV for how receptor binding can be blocked by neutralizing antibodies against the VP1 region; this is unknown for HPeV (green question mark). Pleconaril can bind inside the pocket of HEV **(D)** leading to increased stability of the capsid and conformational changes, thereby interfering with uncoating and cell entry.
VP: viral protein
Figures **(A)** and **(B)** are adapted from [103] with permission from ASM press licence 2010, Figures **(C)** and **(D)** are adapted with permission from a figure courtesy of Dr M Schmidtke (Institute of Virology and Antiviral Therapy, Friedrich Schiller University, Jena, Germany)

6

Receptor interactions & possible determinants for pathogenesis

Human parechovirus types 1, 2, 4, 5 and 6 contain an RGD motif in the C-terminus of VP1 that is utilized by several other viruses for their attachment to cell surface integrins. Among picornaviruses, these include FMDV, CAV9 and echovirus 9 (E9; Barty strain). The RGD motif in HPeV-1 is functional, as demonstrated by blocking experiments with RGD-containing peptides and monoclonal antibodies against αv-integrins, suggesting that αvβ3 and αvβ6 integrins play an important role in the early stages of HPeV-1 infection.[24,31-33] It has been confirmed recently that the binding of both αvβ3 and αvβ6 integrins to HPeV-1 involves the RGD motif in VP1.[25] Furthermore, similar to CAV9, αvβ6 integrin has shown to be a high-affinity receptor for HPeV-1, whereas αvβ3 integrin exhibits lower affinity.[34] Although a few occasional HPeV-1 variants without the RGD motif have been identified,[35] the vast majority of clinical isolates possess this motif. Furthermore, the experimental deletion of the RGD motif from the Harris strain of HPeV-1 was lethal, underscoring its importance in clinical pathogenicity of this virus.[31] By contrast, the RGD motif is important but not essential for the clinical pathogenesis of CAV9 and FMDV.[36-38]

The receptors for the HPeV types that lack the RGD motif in VP1 (HPeV-3 and -7--14) are as yet unidentified.[4,39] How HPeV-3 (and other HPeVs without the RGD motif) enters the cell and whether this contributes to its more severe pathogenicity in humans in comparison to other HPeV types is currently unknown.

Possible animal models to study HPeV infections

Studies on the pathogenesis of HPeV infections have been very limited owing to the lack of suitable animal models. Newborn mice inoculated with HPeV-1 and HPeV-2 were only infrequently infected, while experimentally inoculated cynomolgus monkeys showed no neuropathological changes after the 30 day observation period.[18] Although an experimental mouse model has been developed to investigate the pathogenesis of the rodent-derived LV,[40] it can be estimated that there are likely to be substantial differences in molecular pathogenesis between HPeVs and LVs. HPeVs and LVs are separate species within the genus *Parechoviruses*; they differ from each other genetically as much as, or more, than for example polioviruses do from HRVs. However, HPeV types 1 and 6 have recently been detected in the feces of monkeys with diarrhea.[41] The fecal samples were collected from farmed macaques in China. In this specific case, HPeV infection was suspected to have been transmitted from humans to monkeys and could, thus, potentially serve as an animal model to study the pathogenesis of HPeV infections.

To summarize, HPeV exhibits several distinct molecular features when compared with other picornaviruses. These include the lack of the maturation cleavage of the capsid protein VP0 to VP4 and VP2, a 5’UTR region resembling that of picornaviruses infecting animals and a unique nonstructural 2A protein. The structural differences in particular are relevant to the development of antiviral therapy for HPeV infections (Figure 2), but theoretically every step during the HPeV life cycle from receptor binding to the release of newly formed viral particles is a potential target for viral replication blockage.

HPeV infections & their clinical relevance

Epidemiology

By detection of antibodies to HPeV, 90% of children have shown to be infected with at least one HPeV type by 2 years of age.[42,43] This is confirmed by observations over a time-span of 30 years showing that 60% of 580 HPeV isolates originated from children under 1 year of age.[44] Similarly, surveillance data in the USA between 1983 and 2005 revealed 73% of 456 HPeV-1 infections and 68% of 34 HPeV-2 infections occurred under the age of 1 year,[45] while HPeV-1 and HPeV-3 infections in The Netherlands have been recorded almost exclusively in children under 3 years of age.[35] High incidence of HPeV infections in this age group was also reported in a longitudinal community-based study from Norway, where 11.3% of 1941 fecal samples were HPeV-positive.[46] Interestingly, there are only a few reports in the literature of HPeV infections in individuals over the age of 10 years.[35,47-49] Overall, 90% of the HPeV infections have been described in children younger than 5 years of age,[35,48,50-52] while HEVs generally affect individuals of all ages.

Many HEV surveillance programs still report HPeV-1 and HPeV-2 as echovirus-22 and -23. HPeV-1 is one of the most commonly occurring genotypes when both HPeV and HEV circulation are considered,[53,54] circulating throughout the year but less frequently detected during summer.[12,55] After HPeV-1, HPeV-3 and -6 are the next most frequently detected, depending on the year of isolation and method of screening.[35,47,51,52] HPeV-3 has a biannual circulation pattern and is most frequently found in the summer of even years,[14,35,55] although this specific circulation pattern might be different in other regions of the world.[47] Infections with HPeV-2 are reported sporadically,[55,56] while circulation patterns of the newly reported HPeV types 4, 5 and 7-14 are yet to be determined.

Clinical relevance of HPeV infections

High seroprevalence of HPeV-1 in children and adults indicates that HPeV infections are extremely common and mild or even subclinical, already occurring at a young age. HPeV-1 and -2 were first identified in children suffering from diarrhea.[18] Occasionally, severe conditions such as encephalitis, paralysis, and myocarditis were attributed to HPeV-1 infections (reviewed in Benschop *et al.*[2] and *Harvala et al.*[20]), while infections with HPeV-2 could only be associated with milder symptoms.[56]

Historically, the HPeVs were not considered as separated viral pathogenic entities because they were detected in virus culture designed for HEV diagnostics. With the emergence of molecular techniques HPeVs were no longer diagnosed since PCRs designed for HEV detection were unable to detect HPeVs. With the discovery of the HPeV-3 in 2004,[39] the view on HPeVs changed dramatically, and a new wave of publications increased the knowledge about different HPeV types and their clinical significance (reviewed in Benschop *et al.,*[2] Harvala *et al.,*[4,20] Levorson *et al.*[57]).

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Mild disease: gastrointestinal & respiratory infections

Early reports identified echovirus 22 by cell culture or an increase in numbers of neutralizing antibodies in children with gastrointestinal symptoms or respiratory infections (reviewed in Benschop *et al.*[2] and Harvala *et al.*[20]). WHO data showed that 29% of 581 reported cases of HPeV-1 infections were from patients with gastroenteritis and 26% from patients with respiratory infections.[44] A retrospective investigation of 109 Swedish children with HPeV-1 infection demonstrated diarrhea as the most common clinical finding (32% of cases) followed by respiratory symptoms (13%). HPeV-2 infections have been described in small nosocomial outbreaks of gastroenteritis.[56]

Recent studies report high frequencies (11.6-16%) of HPeV detection in stool samples from children with acute diarrhea.[51,58] In addition, the newly identified HPeV-8 and -10 were first isolated from children with enteritis.[59,60]

Human parechoviruses have been linked to a respiratory disease outbreak on a neonatal unit[4] and associations with upper respiratory tract infections as well as bronchiolitis, pneumonitis and otitis media have been described.[47,48] In a longitudinal follow-up study carried out by Tauriainen *et al.* otitis media and cough were clearly found to be associated with HPeV-1 infections.[61] Conversely, in a screen of 3844 respiratory samples collected in 2007, HPeVs were only detected in 1.2% of the samples.[52] HPeV-1 and -6 have been reported as the most frequently identified types in respiratory specimens.[52] Although HPeV-3 has been reported in association with respiratory disease as well, detection of this type in respiratory samples is scarce.[47,48,52]

Although HPeVs have been diagnosed in a variety of clinical conditions, the detection of HPeV in stool and respiratory samples is not always associated with clinical symptoms. A recent longitudinal study in stool samples from infants showed 11.3% positive for HPeV, irrespective of presence or absence of clinical symptoms.[46] In addition, in the follow-up study carried out by Tauriainen *et al.* no clear association between HPeV-1 infection and gastroenteritis could be found.[61] In addition, approximately 40% of the cases in which HPeV was detected in respiratory specimens originated from children without respiratory symptoms.[52] Indeed, HPeV infections are highly prevalent in children and shedding of viral particles or nucleic acid may occur for weeks (Wildenbeest JG *et al.*, Unpublished Data). Detection of HPeV may therefore represent asymptomatic carriage, even lasting weeks after (symptomatic) infection.

One may assume that the gastrointestinal and respiratory tracts are the primary replication sites of HPeVs. Infection with HPeVs will therefore lead to detection of virus from these sites independent of the presence or nature of clinical symptoms.

CNS infections, neonatal sepsis & other disease associations

Human parechovirus-1 infections were occasionally associated with encephalitis and paralysis,[49,50,62] but less frequently than other echovirus infections.[1,44] Recent reports from The Netherlands, UK and USA have now described HPeV-3 infection with neonatal sepsis

and CNS infections.[12,14,17,63] When HPeV-3 was first characterized, it was isolated from a 1-year-old Japanese girl suffering from transient paralysis.[39] Immediately thereafter, three additional HPeV-3 infections were found in Canadian neonates with neonatal sepsis.[13] The marked clinical difference between HPeV-3 and the two previously known types was initially observed in a Dutch study involving 37 children with an HPeV-1 or -3 infection.[12] Neonatal sepsis was found in 70% of the HPeV-3 infected children and in only 8% of the children infected with HPeV-1. In 50% of the children infected with HPeV-3, CNS-associated symptoms were reported. In comparison to HPeV-1 infections, infections with HPeV-3 were associated with more severe symptoms and with a younger age.[12,55]

Human parechoviruses can be detected as the second most prevalent virus in CSF samples from children.[14,17] By real-time reverse transcriptase-PCR, HPeV was detected in 4.2% of 761 CSF samples from children under the age of 5 years (median age 1.2 months).[17] A total of 75% of the HPeV-positive children presented with sepsis-like illness, whereas symptoms of CNS infection were reported in 16% of these children. Other reported clinical symptoms were gastrointestinal symptoms (39%), respiratory symptoms (36%), and rash (17%). Although in this study HPeV typing from CSF was not performed, the incidence of HPeV positivity in CSF followed the same biannual cycle as noted previously for HPeV-3 from fecal samples,[12,55] suggesting HPeV-3 to be the predominant type to infect the CNS.

In a study from Scotland, comprising 1575 CSF samples from all age groups obtained in 2006-2008, HPeV was detected in 2.6% of the patients, with the highest frequency in 2008 (7.2%) exceeding that of HEVs.[14] All positive samples originated from infants less than 3 months of age with suspected sepsis or pyrexia. Molecular typing of these CSF samples revealed all infections to be due to HPeV-3.

Neonates with HPeV encephalitis exhibit similar clinical symptoms to children with encephalitis caused by HEV infection, the most frequent signs being fever, seizures, irritability, rash and feeding problems.[3] Pleocytosis is found only in a minority of the CSF samples from children with either HEV or HPeV infections, while protein and glucose levels remained normal in all HPeV cases. Normal CSF findings can therefore be misleading when diagnosing neonatal HPeV infection. From the same group, data reported that in ten out of 14 children diagnosed with encephalitis over the last 10 years, HPeV could be detected, mostly typed as HPeV-3.[16] These were all newborn infants presenting with seizures, fever and rash.

Extensive white matter abnormalities with unfavorable neurodevelopmental outcome have been reported in relation to HPeV-3 encephalitis.[16] HPeV-3 has recently also been identified as a cause of neonatal hepatitis-coagulopathy syndrome [63,64] and even infant death.[15]

A number of case reports and small studies propose associations of HPeV with a wide range of other diseases, including myocarditis, hemolytic uremic syndrome, and necrotising enterocolitis (HPeV-1), myositis (HPeV-3), lymphadenitis (HPeV-4) and Reye's syndrome, an acute, noninflammatory encephalopathy with hepatic dysfunction and fatty infiltration (HPeV-5 and -6).[20,47,65] Further studies are needed to confirm these disease associations with HPeV infection.

In conclusion, the clinical spectrum of HPeV infections ranges from asymptomatic infections or mild disease to severe disease symptoms mostly found in young children. In particular, HPeV-3 appears to display a variety of serious clinical presentations including neonatal sepsis, meningitis, encephalitis and hepatitis, and is probably more common than previously anticipated.

Treatment of picornaviruses: limited options

Successful vaccines have been developed against poliovirus, hepatitis A virus and FMDV, viruses from three different genera within the *Picornavirus* family.

Vaccination against poliomyelitis has been successful in eradication of the poliovirus from most parts of the world. However, despite huge efforts by the WHO to eradicate poliovirus world-wide, in 2010 poliovirus is still circulating in India, Afghanistan, Pakistan and Nigeria.[66] It has been suggested that additional antiviral therapy is needed in the polio eradication strategy.[67] Despite long-term efforts, development of antiviral therapy against picornaviruses has not yet been successful and treatment options for human picornaviruses such as HEVs, HRVs as well as HPeVs, are limited.

As illustrated by a recent review on the prognosis of neonates with HEV myocarditis, supportive treatment and administration of intravenous immunoglobulin (IVIg) currently are the only options, with mortality rates of approximately 30% in these conditions.[68] Until mid-2000, the drug pleconaril was occasionally used to treat patients with severe HEV infections. Here, we will review the backgrounds and effects of these treatments against HEV infections to understand potential treatment options against HPeV infection.

IVIg & maternal antibodies in HEV treatment

Neonates are particularly at risk for severe picornavirus infections. Their immune system is not yet fully developed and maternal antibodies derived before birth and during breast feeding play an important role in their host defense. Mothers of neonates with a severe HEV infection frequently had a history of a viral illness preceding or immediately following delivery.[5] In neonates with severe HEV infection, the maternal titers of neutralizing antibodies against the specific HEV serotype were detectable, but generally low, suggesting that a lack of specific maternal antibodies is a risk factor for the development of severe illness.[69]

Another group at risk for severe or chronic HEV infections are patients with primary or secondary immune deficiencies and especially those patients with hypo- or agammaglobulinemia, indicating that a proper humoral immune response is important for HEV clearance.[6] In both groups, lack of (specific) antibodies is associated with severe or chronic infection. This is the rationale to use IVIg as a treatment for severe HEV infections.

In neonates IVIg was used in severe meningoencephalitis, sepsis, hepatitis and/or myocarditis with various clinical outcomes. In the only blinded randomized controlled study 16 neonates with a proven HEV infection were enrolled.[69] Only the neonates (n=5) who had received IVIg with a neutralizing antibody titer of greater than 1:800 against their causative HEV were

able to clear the HEV. However, the study was too small to show statistically significant differences and no effect on clinical outcomes could be found.
Administration of maternal plasma to severely ill HEV-infected infants has been advocated early in infection,[9,70] although it is of note that maternal serum does not always contain high antibody titers against the infecting strain.[69]
The reviews of Crennan *et al.* and Misbah *et al*. describe the effect of IVIg on chronic enteroviral infections in patients with primary immunodeficiencies.[71,72] The use of high-dose IVIg and/or intrathecal immunoglobulins demonstrated variable beneficial effect in patients with chronic enteroviral meningitis in agammaglobulinemia (CEMA). However, the therapeutic efficacy of IVIg in HEV infections has not yet been proven.

The capsid inhibitor pleconaril for treating HRV & HEV
Drugs with capsid-inhibiting properties have demonstrated to be the most promising in the treatment of picornavirus infections. Of these, pleconaril has been evaluated most extensively in clinical trials.
Pleconaril integrates within a hydrophobic pocket inside the viral capsid, leading to increased stability and compression of the viral capsid (Figure 2D). As a result, uncoating and binding of picornavirus to the host cell and of viral RNA are interrupted.[73,74] The hydrophobic pocket is relatively well preserved among HEVs and HRVs, resulting in a broad-spectrum anti-enteroviral and anti-rhinoviral activity.[73] Effectiveness of pleconaril was first shown in the treatment of colds due to picornaviruses in adults[75] and the efficacy of pleconaril has been summarized in several reviews.[6,74,76] The effect of pleconaril in neonates with severe HEV infection varied[77] and effects on recovery of HEV meningitis was minor.[78] Pleconaril has been used as treatment on a compassionate-use basis in patients with immunodeficiencies and severe HEV infections, very often in combination with IVIg. In a group of 17 immunoglobulin-deficient patients with CEMA treated with pleconaril for 7-10 days, 12 patients (75%) showed a clinical response to therapy.[77] Further support for the benefit of pleconaril in immunocompromised patients is anecdotal,[79,80] while cases with fatal outcome also have been described.[81] In our hospital, a child with CEMA with an echovirus-13 infection cleared the virus from the CSF after treatment with pleconaril and IVIg (Wildenbeest JG *et al.*, manuscript in preparation).
Later investigations revealed that pleconaril induces hepatic cytochrome P450 3A enzymes, leading to menstrual irregularities and therefore risk of unplanned pregnancy in women who used oral contraceptives. This, and other concerns about possible drug interactions and resistance resulted in the rejection by the US FDA in 2002 of use of pleconaril as a treatment for the common cold.[76] Thereafter, production of pleconaril was abandoned and the drug is no longer available.[74]
In summary, although the efficacy of pleconaril could not inconclusively be demonstrated for all indications, it was the only antiviral compound ever to be available for treatment of severe HEV infections. Based on the structure of pleconaril, other capsid-inhibiting

compounds are being developed with the emphasis of activity against EV-71, which was resistant to pleconaril.[82-84] In addition, compounds targeting the protease are being designed such as 3C protease inhibitors against EV-71 (Figure 2A).[76,85]

Expert commentary: the need for development of anti-HPeV therapy

Current options

The need for therapy against HEVs and HRVs has been emphasized in numerous reviews and studies over the last decade.[6,74,76] HPeV infections can be severe and even life-threatening, indicating a need for treatment. So far, no systematic data are available on HPeV treatment. In a case report describing a twin with neonatal sepsis and hepatitis infected with HPeV-3, one child received IVIg and subsequently recovered, while the other recovered having received acyclovir which obviously does not have antipicornaviral activity.[64]

If IVIg is given to neonates to reduce disease burden from HEV infection, it seems rational to give IVIg to severely ill neonates with HPeV infection as well. High antibody titers against the specific serotype might be needed for protection.[69] Neutralizing antibody titers in IVIg vary between batches [86] and geographic regions,[87] but the high seroprevalences of HPeV-1 and -3 in adults would suggest IVIg to contain high titers of neutralizing antibodies against these HPeV types.

Another option that should be explored for treatment of HEV and HPeV infections is the use of monoclonal antibodies. New approaches to rapidly generating human monoclonal antibodies have been successful in the development of monoclonal antibodies against influenza viruses [88] and respiratory syncytial virus.[89] For HEV, protective antibodies are presumably neutralizing type-specific antibodies against the VP1 capsid protein; therefore, monoclonal antibodies against HEV will not exhibit broad cross-neutralizing capacity, as recently described for influenza virus. This is a problem when considering generating monoclonal antibodies for the treatment of HEV infections, with over 100 serotypes and multiple serotypes circulating at the same time without a clear type-dependent disease association. By contrast, for HPeV this approach could be feasible. The HPeV group is much smaller and neutralizing antibodies elicited against VP0 showed cross-reactivity.[26] Furthermore, HPeV-3 stands out for its association with more severe disease, making it an ideal target for monoclonal antibody neutralization.

Although there is circumstantial evidence for protection of antibodies against severe disease in HEV infections, this has never been shown for HPeVs and is questioned by Ehrnst and Eriksson.[50] They observed that, despite the presence of maternal antibodies in almost all mothers in their study, symptomatic infection with HPeV-1 occurred in infants that still should have maternal antibodies present. In addition, symptomatic HPeV-3 infection in infants occurs at a very young age, arguing against maternal protection by antibodies, although one could argue that infants with severe HPeV-3 infections were all born from

HPeV-3 seronegative mothers. Therefore, the potential of antibodies to protect against or to reduce symptomatic HPeV infection still needs to be determined.
Currently, the antibody approach seems to be the only one available for treatment of HPeV infections.

Potential options

Theoretically, every step in the viral life cycle is a potential target for developing antiviral therapy. An extensive overview of compounds that can inhibit picornavirus replication is given by de Palma *et al.*[76] Although this review is quite recent, no data can be found on compounds that can potentially inhibit HPeVs. The most promising candidates for anti-picornaviral therapy propagated in the literature are capsid inhibitors and 3C protease inhibitors (Figure 2).[76,82,83,85]
As described in more detail earlier in this article, pleconaril was the most promising capsid-inhibiting compound with almost all criteria for a good antipicornaviral drug present: *in vitro* and *in vivo* activity and clinical activity shown for some patient groups, combined with a favorable safety profile. A major difference between HPeVs and HEV/HRV is that the capsid consists of three structural proteins rather than the four typically seen.[2] Therefore, capsid stability and infectivity must be differentially regulated in HPeVs. The 3D structure of HPeV does have similarity to some other picornaviruses (most closely to FMDV), despite very limited amino acid sequence identity.[25] The external appearance of HPeV-1 particles is much smoother than other picornaviruses, such as CAV9, most likely due to truncated surface loops of VP1. This could indicate that the hydrophobic pocket differs from that of HEVs, possibly preventing the activity of pleconaril. Indeed, data from our laboratory show that HPeV-1 and -3 are resistant against pleconaril.[4] (Wolthers KC *et al.*, Unpublished Observation) Interestingly, Holmberg *et al.* used pleconaril for treatment of mice and rats infected with LV, another member of the *Parechovirus* genus.[90] Minor inhibitory effects of pleconaril on LV in cell culture growth were described but a CPE inhibition test was not performed and IC_{50} values were not given.
Several capsid-inhibiting compounds are being developed against HRV and HEV, some with the emphasis on targeting the pleconaril-resistant CBV3 or HEV-71.[76,84] Given the different structure of the HPeV viral capsid, these HEV/HRV capsid-inhibiting compounds may not inhibit HPeV capsid functionality, although this could easily be tested *in vitro*.

The protease 3Cpro is ubiquitous in the picornavirus family and for HPeVs it seems to be the only protease.[1] For the 3C protease inhibitor rupintrivir, antiviral activity was shown in a human experimental HRV challenge trial, where disease severity and viral load were reduced;[76] however, no reduction of these parameters could be found in naturally infected patients, and the clinical development of rupintrivir was halted. Despite this, rupintrivir was recently promoted for treatment of severe EV-71 infections.[91] The 3C protease inhibitor compounds have a broad anti-picornaviral activity.[76] It is uncertain whether rupintrivir would

have had any inhibitory effect on HPeVs; although the genome structure of HPeVs is similar to HEVs, the genome variability is extensive, and despite conservation of the 3CD regions, structure and function of the HPeV 3C protease might differ from other picornaviruses.
Human parechoviruses can be cultured *in vitro* on standard cell lines; HPeV-1 and -2 can easily be propagated on many cell lines used in the laboratory, while HPeV-3 grows slower and only on a limited amount of cell lines. Thus, susceptibility of HPeVs to different antiviral compounds can be tested *in vitro* just as well as for HEVs and HRVs. Antiviral effect and cytotoxicity of pleconaril and related compounds have been studied for CVB3 by measuring cell viability and inhibition of cytopathic effect in cell culture.[84] Cell culture models are also used in high-throughput screening of antiviral compounds as recently presented.[92,93] Including HPeV-1 as the prototype HPeV and HPeV-3 as the most pathogenic HPeV type in these screenings would be a step further in development of an anti-HPeV treatment.

Highly speculative options

In a recent study, ribavirin was shown to have *in vitro* and *in vivo* effectivity against EV-71 in a mouse model and it was suggested that ribavirin could be a potential drug for EV-71.[94] Of note, Holmberg *et al.* used ribavirin in addition to pleconaril in the treatment of rats and mice infected with LV.[90] Ribavirin is a nucleoside analogue with broad-spectrum antiviral activity, currently used to treat patients infected with hepatitis C and occasionally in patients with Lassa fever. Ribavirin acts by different mechanisms to inhibit virus replication. One mechanism of action described for poliovirus is lethal mutagenesis, which is the loss of infectivity with an increase in mutation rate.[95] Passaging poliovirus in the presence of ribavirin leads to a viral population resistant to ribavirin, but these viruses are less adaptable, making them more susceptible to other antiviral drugs. This would be a challenging approach for combination therapy, but the mutagenicity of ribavirin will also make it difficult to get the drug approved for use in human picornavirus infection.
Recent investigations explore the role of RNA interference in inhibiting replication of picornaviruses such as EV-71 and CAV-21.[96,97] By targeting virus gene regions or host factors critical for viral replication by small interfering RNAs, virus replication can be suppressed, indicating that this is a promising approach for developing antivirals. At this time, RNAi seems much more a tool that could be applied for research on HPeVs than for treatment development.

Five-year view

Compared with HEVs, less is known about receptor use, replication pathways, viral pathogenesis, or virus-host interactions of HPeVs.[1,2,4] More research is needed to elucidate the specific characteristics of this clinically relevant group of viruses and to develop treatment strategies. In the meantime, the HPeVs should be included in the ongoing search for antiviral

compounds against picornaviruses. Although the withdrawal of pleconaril has been a major setback for the treatment of picornavirus infections, many promising compounds are designed and tested against several picornaviruses. However, it may take years before this will lead to a candidate drug that can be tested in the clinic. If such a compound does not have a broad-spectrum activity against picornaviruses, including the HPeVs, than for the latter group, development of an antiviral compound may take much longer. Antibody-based therapies therefore seems to be the most feasible as a short-term option for treating HPeV infections.

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Key issues

- The group of Human Parechoviruses (HPeVs) belong to the family of *Picornaviridae* and are closely related to the well-known group of the human enteroviruses (HEVs), a large group of viral pathogens that comprise the most common causes of human infections.
- The clinical profile of HPeVs overlaps with that of HEVs, ranging from mild febrile disease to neonatal sepsis, CNS infections and hepatitis.
- The HPeV group contains 14 HPeV genotypes, of which HPeV-3 is associated with neonatal sepsis and CNS infections in younger children compared with infections with other HPeV types.
- No antiviral treatment is currently available against HPeVs or HEVs.
- Intravenous immunoglobulin could be of help for treatment of severe HPeV infections; intravenous immunoglobulin is sometimes used to treat severe HEV infections, based on the observation that lack of serotype-specific antibodies can lead to severe or chronic HEV infection.
- Development of specific HPeV antibodies for treatment of HPeV infections could be a feasible approach for the near future.
- Capsid inhibitors are currently under development for antipicornaviral therapy, of which pleconaril is the best studied. Pleconaril will most likely not have antiviral activity against HPeVs; and also, this drug is no longer available.
- The two most promising approaches to development of antipicornaviral therapy are capsid inhibition and protease inhibition. Compounds in development should be tested for antiviral activity against HPeVs.

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Chapter 7

Pleconaril revisited: clinical course of chronic enteroviral meningoencephalitis after treatment correlates with *in vitro* susceptibility

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Abstract

Background

Human enteroviruses (HEVs) can cause severe infections, especially in patients with a deficient humoral immune response, such as X-linked agammaglobulinemia. In this patient group, chronic enteroviral meningitis (CEMA) is feared because of extensive morbidity and high fatality rate. Treatment options consist of intravenous immunoglobulin (IVIG), with various outcomes. Pleconaril is an antiviral agent with *in vitro* activity against HEVs that has been used in the treatment of HEV infections.

Methods

The efficacy of pleconaril and IVIG against HEV isolated from the patients was assessed *in vitro* in two patients with CEMA.

Results

Echovirus 11 was found in the cerebrospinal fluid (CSF) of case 1. Treatment with high-dose IVIG and pleconaril did not provide any clinical improvement and HEV PCR in CSF remained positive. Case 2 (echovirus 13 positive in CSF) was also treated with IVIG and pleconaril. The patient recovered completely and HEV PCR in CSF became negative. Recent IVIG batches contained low titers of neutralizing antibodies against the patient strains. Echovirus 11 (case 1) was resistant to pleconaril *in vitro,* whereas echovirus 13 (case 2) was susceptible, in accordance with virological response after treatment and subsequent clinical results.

Conclusions

This is the first report that evaluates efficacy of antiviral treatment in CEMA patients in relation to *in vitro* susceptibility of clinical virus isolates. Since pleconaril is no longer available for compassionate use we strongly propagate that new drugs should be developed against these potential life threatening HEV infections.

Introduction

Human enteroviruses (HEVs) are single-stranded, positive-sense RNA viruses within the *Enterovirus* genus of the large *Picornaviridae* family. The genus *Enterovirus* contains >200 different serotypes known to infect humans, including polioviruses, echoviruses, Coxsackie A and B viruses, the numerically identified enteroviruses 68 to 109, and rhinoviruses.

The immunological defence against HEVs including poliovirus greatly depends on the presence of neutralizing antibodies (nAbs).[1] Therefore groups of patients with a deficient humoral immune response, like newborns and patients with primary or secondary immunodeficiencies are particularly susceptible to severe infections with HEVs. A severe and much feared condition in patients with primary immunodeficiencies, most particular in X-linked agammaglobulinemia (XLA), is chronic enteroviral meningoencephalitis (CEMA).[2,3]

XLA is a rare immunodeficiency due to an arrest in early B-cell differentiation, caused by mutations in the Bruton tyrosine kinase gene. This leads to severe hypogammaglobulinemia and markedly reduced B-cells.[4] This condition makes patients with XLA particularly susceptible for disseminated or chronic HEV infections. The mortality in this group of patients is considerable with 35% of deaths due to disseminated HEV infections as was shown in a registry of patients with XLA in the United States.[5-9]

Since the introduction and regular use of intravenous immunoglobulin (IVIG) in the treatment of humoral immune defects, the incidence of CEMA has decreased, although IVIG can not prevent all cases.[5,9,10]

Treatment options for severe HEV infections are limited. In most patients treatment with IVIG is given, sometimes combined with intrathecal administration of immunoglobulins in the case of CEMA.[2,3,10,11] Currently, no antiviral drugs are available against HEV infections. In the past, the antiviral compound pleconaril has been used on a compassionate-use basis in patients with severe HEV infections.[12] Pleconaril blocks the binding of the enterovirus to cells by interfering with the capsid proteins of the virus and shows *in vitro* antiviral activity against most HEVs and rhinoviruses.[13] The uptake and bioavailability of pleconaril is excellent, even in the cerebrospinal fluid (CSF).[13] No serious adverse events were noted in treated patients.[14,15] Currently, pleconaril is no longer available for compassionate use. However, the medical need for treatment in some patients with severe HEV infections can be urgent. We here describe two patients with CEMA that failed therapy with IVIG who were treated with pleconaril as a last resort. Our report is the first to correlate the clinical response in two CEMA patients with *in vitro* susceptibility testing of the infecting strains for pleconaril and IVIG. Our results will be put in perspective of previously published literature, further illustrating the need for antiviral treatment in severe HEV infections.

Methods

Virus detection, isolation and typing

HEV was detected by real-time reverse transcriptase PCR in CSF samples, stool samples, throat swabs, and EDTA blood samples from the patients as previously described.[16]

Virus isolation was performed by co-cultivation of PCR-positive CSF samples on different cell lines in 24-wells plates.[17] Virus growth was observed by cytopathogenic effect (CPE). Culture isolates were characterized by serotyping with HEV-specific horse antisera pools A-G and H-R (RIVM, Bilthoven, The Netherlands).[18] For genotyping of the culture isolates of the first patient, the nested approach as described by McWilliam Leitch *et al.*[19] was used, while for genotyping of the culture isolates of the second patient, the seminested approach by Nix *et al.*[20] was used, resulting in a 350-400 base pair sequence fragment of the VP1 gene. The VP1 sequence of the culture isolate was compared to VP1 sequences of HEV reference strains and phylogenetically characterized based on cluster analyses.

End point neutralization assay

Presence of nAbs in IVIG as well as serum obtained from case 2, was tested by neutralization assay. Only the IVIG that case 2 had received was available; case 2 had received two different batches of IVIG, including IVIG (batch 1: Sanquin, Amsterdam, the Netherlands) and Nanogam® (batch 2: Sanquin, Amsterdam, the Netherlands). Serial 10-fold dilutions of virus isolates in concentrations ranging from 50% tissue culture infectious dose ($TCID_{50}$) of 10^0–10^6 were incubated in 96-well plates with two-step dilutions of the IVIG batches or serum (undiluted, 1:5 - 1:20,480). Cell cultures were incubated for 7 days at 37°C and 5% CO_2, and CPE was scored. Titres of nAbs were calculated by end-point neutralization.

Cytopathogenic effect inhibition assay

The 50% inhibitory concentration (IC_{50}) of pleconaril against echovirus 11 and 13 isolated from the 2 patients was calculated from a cell culture assay measuring inhibition of virus CPE by different concentrations of the compound.[13,21] Coxsackievirus A9 (CAV9), Rhinovirus 16 (RV16; a kind gift of Koen F van der Sluijs, Laboratory of Medical Immunology, AMC, Amsterdam, the Netherlands) and human enterovirus 71 (HEV71) culture isolates were included as controls. Virus isolates in Eagle's modified essential medium and 2% fetal calf serum were incubated at different concentrations (100, 200 and 500 $TCID_{50}$/50 µl) on 96-wells plates with a monolayer of cells (HT29 or Hel cells). Pleconaril was obtained from Sequoia Research Products Ltd (Pangbourne, UK). Pleconaril was tested in serial 10-fold dilutions (100-0.0001 µg/ml) in quadruplicate. Pleconaril was solubilized in dimethyl sulfoxide (DMSO) 40 mg/ml as described by Pevear *et al.*[13], and pleconaril/DMSO solutions were prepared so that the DMSO end-concentration was the same in every well.[13] Each plate included controls for virus growth, toxicity of DMSO on virus growth, toxicity of DMSO on cells, and cell growth. Cell cultures were incubated for 8 days at 37°C and 5% CO_2, and CPE was scored. IC_{50} was calculated using the Reed and Muench[22] method.

Results

Case 1 clinical course

The first patient was a man born in 1968, and in that same year XLA was diagnosed. This patient had a history of psychiatric problems starting at the age of 14, with episodes of neurological complaints. An enterovirus was cultured from the CSF in 1986, for which the patient received increased doses of IVIG selected for high titers against HEV. A liver biopsy in 2005 showed a chronic active hepatitis for which no cause was found.

In September 2007 the general condition of the patient was poor, with extreme fatigue and fever. The CSF showed 208 cells/mm^3 and a total protein of 0.91 g/l. By PCR, HEV was detected in the CSF and in blood. The patient was treated with 25 g IVIG per week resulting in immunoglobulin (Ig) G levels in the blood of approximately 14 g/l. The clinical situation stabilized. In June 2008 an MRI scan showed quadriventricular hydrocephalus with transependymal oedema. In October 2008 treatment with pleconaril 400 mg three times daily for 10 days was started (Figure 1A). The therapy was well-tolerated, but there was no clinical improvement; 1 month after treatment, CSF PCR was still positive for HEV. The IVIG dose was increased to 30 g per week resulting in blood IgG levels around 18 g/l. Until October 2010 the condition of the patient remained the same without evident progression of neurological disease. In April 2010 he was admitted to the Psychiatry department for severe depression; 6 months later there was a sudden deterioration with a right-sided paresis, confusion, impaired consciousness and fever. After a few days, these symptoms disappeared. The CSF showed 132 cells/mm^3, a total protein of 1.05 g/l and, again, a PCR positive for HEV. MRI showed no improvement compared to June 2008.

Case 2 clinical course

The second patient, a 12-year old boy with XLA for which he was treated with IVIG every 4 weeks, was doing well until September 2008. At that time he was admitted to the hospital because of headache, dizziness, nausea and vomiting, together with walking difficulties. He had no fever, C-reactive protein was <1 mg/l, and his levels of IgG, IgA and IgM were 6.0, < 0.01 and <0.1 g/L, respectively. Neurologic examination showed ataxia and an MRI scan of the brain revealed a quadriventricular hydrocephalus and a diffuse enhanced signal of the meninges. The CSF showed a strongly elevated protein level (6.28 g/L), leukocytes of 170 cells/µL and red blood cells of 113.000 cells/µL. By PCR, HEV was detected in the CSF. Stool samples, EDTA blood and throat swabs remained negative for HEV. No other pathogenic microorganism was detected.

An Ommaya drain was placed because of the hydrocephalus. IVIG therapy was intensified to 18 g (0.5 gram/kg) every 3 weeks from the beginning of October, resulting in plasma levels of IgG of ≥9 gram/l. Because of persistent symptoms of ataxia, headache and nausea, treatment with pleconaril was started in November 2008 (Figure 1B). The drug was given orally at a dose of 600 mg per day (17 mg/kg/day), divided in three doses for 2 weeks.

The patient had no side effects of the antiviral compound, but the symptoms of nausea and ataxia initially did not improve. In December 2008, the boy was readmitted because of worsening of symptoms due to an increased hydrocephalus. A ventriculoperitoneal drain was inserted. At the same time, weekly IVIG from another batch (batch 2; Nanogam, Sanquin; 0.5 g/kg; Figure 1B) was started. The neurological symptoms improved rapidly and the patient was discharged from the hospital. In the following month, the nausea, headache and ataxia disappeared completely.

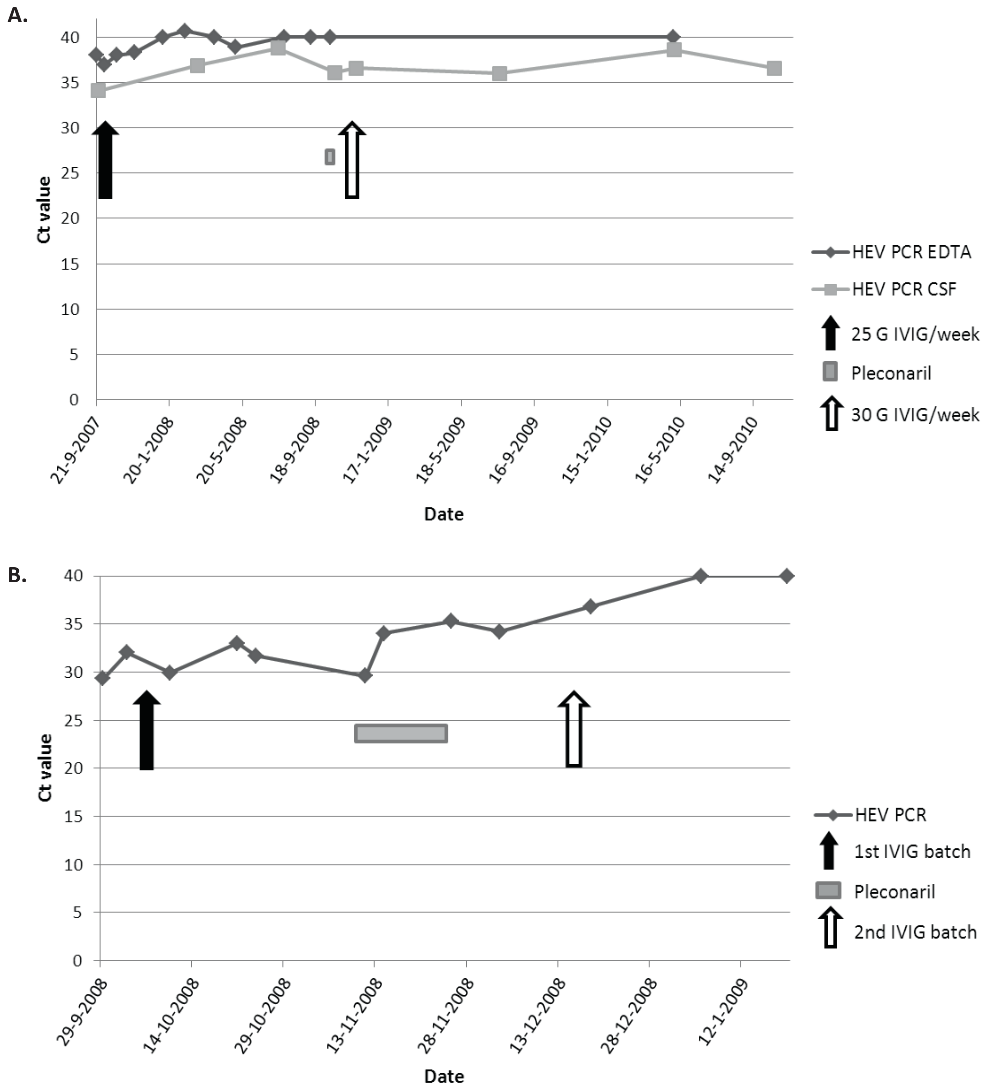

Figure 1. Effect of treatment with IVIG and pleconaril on PCR detection of enterovirus.
Cycle threshold (Ct) value in relation to intravenous immunoglobulin (IVIG) and pleconaril administration in **(A)** case 1 and **(B)** case 2. A Ct value >40 is undetectable. CSF, cerebrospinal fluid; HEV, human enterovirus.

Virological response to treatment

Virological response measured by the cycle threshold (Ct) value of the real-time PCR is shown in Figure 1.

In case 1, the initial Ct value in CSF was 34.1, indicating low viral load (Figure 1A). After intensifying treatment with IVIG, the Ct value in the liquor seemed to increase, indicating a decrease in viral load, but a year later the viral load was around the same value as initially. Treatment with pleconaril in October 2008 did not give any change in CSF viral load and, since then, the viral load stayed in the same range despite further intensification of treatment with IVIG. The viral load in EDTA blood was initially low (Ct value 38) and became undetectable from 2008 except for one Ct-value of 38.9 in May 2008.

In case 2, the initial Ct value in CSF was 29.3, indicating moderate-low viral load (Figure 1B). Despite intensifying treatment with IVIG, the viral load in CSF remained in the same range. After start of treatment with pleconaril, the Ct value increased, indicating a decrease in viral load. After the pleconaril was stopped, initially the Ct value seemed to stabilize, but follow-up after readmittance and administration of Nanogam indicated a persistent decrease with subsequent undetectable viral load (Ct values >40) in January, which coincided with clinical improvement.

In vitro susceptibility for IVIG and pleconaril

In both cases, virus could be isolated from the CSF by cell culture. By serotyping, the culture isolates from case 1 and 2 could be identified as echovirus 11 and echovirus 13, respectively. Serotyping was confirmed by VP1-genotyping of the culture isolates.

Susceptibility of different virus strains to neutralization by the available IVIG batches was tested *in vitro* (Table 1). Both IVIG batches showed moderate to high nAb titres against CAV9, RV16, HEV71 and human parechovirus type 1 (HPeV1). However, very low nAb titres (<32) could be detected against echovirus 11 and 13 obtained from the patients. In addition, three sera from case 2 were tested for neutralizing capacity against echovirus 13 and HPeV1 after IVIG administration. None of the sera could neutralize the patient's echovirus 13, while moderate to high nAb titres against HPeV1 could be found, in agreement with the level of nAbs titres in both IVIG batches (Table 1).

Susceptibility for pleconaril of the echovirus 11 and 13 isolated from the patients was tested by calculation of the IC_{50}. As positive controls, the viruses CAV9 and RV16 were tested and were found to be susceptible to pleconaril *in vitro*, with IC_{50} values in concordance with previous results;[13,21] HEV71 was included as negative control and indeed was resistant against pleconaril.[23] IC_{50} against pleconaril of both strains of echovirus 11 obtained from case 1 indicated that these strains were not susceptible. However, the IC_{50} of the strain of echovirus 13 obtained from case 2 was comparable to the susceptible control viruses (Table 1).

Table 1. Susceptibility of enterovirus isolates for neutralization by IVIG and inhibition by pleconaril.

	nAbs in IVIG[a]		nAbs in serum of case 2[a]			Pleconaril IC_{50},
Virus type	**Batch 1**	**Batch 2**	**28-09-08**	**3-12-08**	**16-3-09**	**µg/ml**
Echovirus 13[b]	20	10	<8	<8	<8	0.032
Echovirus 11 (2007)[c]	8	ND	ND	ND	ND	>32
Echovirus 11 (2010)[d]	32	ND	ND	ND	ND	>32
CAV9	1,280	1,280	ND	ND	ND	0.032
RV16	320	160	ND	ND	ND	0.022
HEV71	640	320	ND	ND	ND	>100
HPeV1	10,240	1,280	256	128	512	ND

[a]Titre at a concentration of 100 50% tissue culture infective dose. [b]Echovirus 13 obtained from case 2. [c]Echovirus 11 obtained from case 1 in the year 2007. [d]Echovirus 11 obtained from case 1 in the year 2010. CAV9, Coxsackievirus A9; HEV71, human enterovirus 71; HPeV1, human parechovirus type 1; IC_{50}, 50% inhibitory concentration; IVIG, intravenous immunoglobulin; nAbs, neutralizing antibodies; ND, not determined; RV16, Rhinovirus 16.

Discussion

We here present the first report describing two cases of CEMA in which the efficacy of treatment with pleconaril and IVIG was verified *in vitro* on the virus isolated from the patients. We showed *in vitro* that recent IVIG batches contained very low nAb titers against echovirus 11 and 13 isolated from our patients, in accordance with absence of clinical improvement in both patients after intensifying treatment with IVIG.

We also showed that the echovirus 11 isolated from the first patient was resistant against pleconaril while the echovirus 13 from the second patient was sensitive to pleconaril in accordance with clinical observations.

Pleconaril has been used as treatment on a compassionate use basis in patients with immunodeficiencies and severe HEV infections. In a group of 17 patients with CEMA treated with pleconaril for 7-10 days, 75% showed a clinical response to therapy, while no serious adverse events were seen.[12] In an adult case of chronic echovirus 13 meningoencephalitis, which persisted despite high doses of IVIG, the virus was cleared after treatment with pleconaril and the patient recovered, in agreement with our data.[24] Further support for benefit of pleconaril in immunocompromised patients is anecdotic (Table 2), while cases with fatal outcome also have been described.[25-31] In none of the severe HEV cases treated with pleconaril has its effect been properly evaluated; sometimes, HEV infections were not even proven,[12] or the infecting serotype was unknown.[25-28] Furthermore, pleconaril was almost always given in combination with IVIG, while the *in vitro* susceptibility of the infecting strains for IVIG and pleconaril was never tested in these clinical cases.

To our surprise, both echovirus 11 strains of case 1 were resistant to pleconaril, while laboratory strains of echovirus 11 were highly susceptible.[13] Resistance could be due to adaptation of the virus and the forming of resistant strains in reaction to treatment with

pleconaril, but our patient was never treated with pleconaril or any other antiviral therapy before 2008. Several other family members had agammaglobulinemia, but none of them was ever treated with pleconaril. For CBV3, pleconaril resistance has been described in various laboratory and clinical strains due to a polymorphism in the hydrophobic pocket amino acid sequence.[32] Between echovirus 11 strains, a large nucleotide variation can be found, dividing them in genogroups;[33,34] therefore, it could be that some echovirus 11 strains carry polymorphisms leading to pleconaril resistance.

The role of IVIG in the treatment of patients with CEMA has not been clear. Variable results have been reported about the use of high-dose IVIG in CEMA, sometimes combined with intrathecal immunoglobulins, as reviewed by Misbah *et al.*[10] and Crennan *et al.*[11] In CEMA patients with echovirus 11 infection who were treated with IVIG, complete recovery as well as death have been reported.[10] In one case, the echovirus 11 was neutralized *in vitro* with the IVIG batch the patient received, but *in vivo* the virus was still found in the CSF of the patient, despite high doses of IVIG and intrathecal immunoglobulin administration.[10] This suggests that the *in vivo* concentration of nAbs in CSF was still not high enough to eliminate the virus.

Earlier studies on maternal antibodies indeed suggested that only high antibody titres correlate with protection against disease.[35] We found low nAb titre against echovirus 11 in recent IVIG batches. Although the IVIG from case 1 was not available for testing, relatively low echovirus 11 nAb titres have been found in older IVIG batches as well, while echovirus 11 was one of the most frequently isolated HEV during that period.[36]

The genetic variation of circulating echovirus 11 strains could lead to antigenic differences [33,34] in which nAbs against one genogroup do not neutralize other genogroups.

As shown for echovirus 11, the nAbs in the IVIG batches were low for echovirus 13 as well. In contrast to echovirus 11, isolation of echovirus 13 before the year 2000 was rare and it is considered an emerging cause of HEV meningitis in several countries during the past decade.[24,37,38] Therefore, it could be that echovirus 13 nAbs are not yet highly prevalent in adult blood donors. Alternatively, as for echovirus 11, antigenic variation could prevent neutralization of specific genogroups by nAbs against other genogroups.

During clinical follow-up, the virological response of case 2 after administration of Nanogam suggested that this second IVIG batch had been effective in clearing the virus from the CSF; however, both first and second IVIG batches contained low echovirus 13 nAb titres and no echovirus 13 nAbs could be found in the blood of the patient, indicating that it must have been the pleconaril that cleared the virus.

Table 2. Overview of case reports about treatment with pleconaril in patients with immunodeficiencies.

Reference	Age	Underlying disease	Symptoms	Virus (site of isolation)	Duration of pleconaril (time after initial symptoms)	IVIG given (time after initial symptoms)	Outcome
Quartier *et al.*[25]	9 Years	XLA	CEMA	Enterovirus (CSF)	7 Days	Prophylactic every 3 weeks, intensified during symptoms	CR (2 months after pleconaril)
	14 Years	XLA	CEMA, hepatitis	Echovirus 11 (CSF, blood, urine, stool)	7 Days (2nd month); 14 days (8th month)	Prophylactic, intensified during symptoms; Intraventricular Ig (11th month)	CR (from 16th month)
Archimbaud *et al.*[24]	53 Years	Lymphoma relapse with immunosuppresive therapy	Meningoencephalitis	Echovirus 13 (CSF)	10 Days (8th month)	Yes, for 12 months (7th month)	CR (few weeks after pleconaril)
Nowak-Wegnzyn *et al.*[26]	11 Months	SCID	Chronic diarrhoea, failure to thrive	Enterovirus (stool)	7 Days	No	Diarrhoea resolved (HEV-negative), died of haemolysis (unrelated to pleconaril)
	9 Months	Omenn syndrome	Chronic diarrhoea, failure to thrive	Enterovirus (stool)	7 Days	No	CR of diarrhoea
Starlin *et al.*[29]	39 Years	Cystic fibrosis with renal and lung transplantation with immunosuppressive therapy	Acute flaccid paralysis, meningitis	Echovirus 19 (CSF, blood)	10 Days (4th day)	Yes, for 5 days (4th day)	CSF negative for HEV, symptoms improved, died of respiratory failure after 2 months
Schmugge *et al.*[28]	26 Years	XLA	CEMA	Enterovirus (CSF)	10 Days (18th month)	Prophylactic every 4 weeks, intensified during symptoms	CR
Tormey *et al.*[27]	18 Years	CVID	CEMA	Enterovirus (CSF)	10 Days (after diagnosis was made); 10 days (4th month)	Prophylactic every 3 weeks	CR (after 2nd course of pleconaril)
	44 Years	AIDS	Meningoencephalitis	Enterovirus (CSF)	10 Days (after diagnosis was made)	No	CR
Katsibardi *et al.*[30]	18 Years	CVID, Hodgkin lymphoma (in remission)	IAHS	Coxsackie B3 (blood, throat, urine, feces)	10 Days (after diagnosis was made)	Yes, single dose (after diagnosis was made)	Died of MOF (virus PCR-negative)
	11 Years	Non-Hodgkin lymphoma (in remission)	IAHS	Coxsackie B3 (blood, throat, urine, feces)	3 Days until death (after diagnosis was made)	Yes, single dose (after diagnosis was made)	Died of MOF 3 days after start pleconaril
Cree *et al.*[31]	33 Years	History of Henoch-Schönlein purpura, juvenile RA treated with immunosuppressive therapy and haemodialysis for IgA nephropathy	Meningoencephalitis, myocarditis	Coxsackie B4 (CSF, rectum)	7 Days (around 1 month)	No	Died at 53rd day after onset of symptoms

CEMA, chronic enteroviral meningoencephalitis in agammaglobulinemia; CR, complete recovery; CSF, cerebrospinal fluid; CVID, common variable immunodeficiency; HEV, human enterovirus; IAHS, infection-associated haemophagocytic syndrome; Ig, immunoglobulin; IVIG, intravenous immunoglobulin; MOF, multiorgan failure; RA, rheumatoid arthritis; SCID, severe combined immunodeficiency; XLA, X-linked agammaglobulinemia.

There are no precise data about the time needed to reach clinical and/or virological response for pleconaril, although in general viral clearance has been reported to occur within 2 weeks after treatment.[24,26,28,29] It could be that our method of detection by real-time PCR is much more sensitive than previously used detection methods, resulting in a much longer time before viral clearance can be shown. In accordance with our results, Quartier *et al.*[25] suggested a delayed effect of pleconaril in two juvenile patients with CEMA who were treated with IVIG and pleconaril.
In 2002 the FDA rejected the use of oral pleconaril against the common cold.[39] Currently, pleconaril is no longer available for use on compassionate basis in severe HEV infections. However, the great variety of clinical symptoms caused by different HEV strains in combination with insufficient follow-up of its antiviral effects may have contributed to earlier inconclusive results.

Conclusion

We here show that antiviral therapy against chronic enteroviral infections is necessary and can be clinically effective if the HEV strain is susceptible. Although this may seem obvious, clinical results with pleconaril in severe HEV infections have not been evaluated using our methods, and contradictory results may have been the results of a lack of *in vitro* data. Therefore, more cases are needed to further determine the possibilities of *in vitro* testing of response to antiviral treatment in patients with severe viral infections. We therefore strongly propagate that new drugs should be developed against these severe HEV infections, and that these drugs should be evaluated *in vitro*, not only during development, but also during clinical evaluation, taking into account the variety of clinical syndromes as well as the biological and genetic variety of the circulating HEV strains.

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Chapter 8

Genetic and antigenic structural characterization for resistance of Echovirus 11 to pleconaril in an immunocompromised patient

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Abstract

Pleconaril is a capsid-inhibitor previously used to treat enterovirus infections. A pleconaril-resistant Echovirus (E)11 strain was identified before pleconaril treatment was given in an immunocompromised patient. The patient was also treated with intravenous immunoglobulin (IVIG) for a long period, but remained unresponsive. The intrinsic resistant strains could not be neutralized confirming IVIG treatment failure. To identify the basis of the intrinsic resistance, genetic and structural analyses were conducted. Analysis of the viral capsid structure indicated structural changes of the hydrophobic pocket that disabled pleconaril to efficiently dock within the pocket. Substitutions V117I, V119M, and I188L were found in the pocket region. Both V119M and I188L were found to affect the structure the pocket. However, by reverse genetics, V119M was shown to confer pleconaril resistance, whereas I188L did not confer resistance on its own. In contrast, pleconaril resistance induced *in vitro* in a susceptible E11 clinical isolate was underlined by a single I183M substitution conferring resistance through inefficient docking of pleconaril by the protruding sulphate side chain of methionine rather than structurally changing the pocket.

In conclusion, we showed that intrinsic resistant strains display different markers with different mechanisms than identified *in vitro*; this should be taken into account when evaluating resistance profiles of new drugs *in vitro*. These data indicate multiple factors to have played a role in pleconaril resistance in the patient strain. Based on IVIG treatment failure of the patient we predict one of these factors to be immune-related. Both IVIG and capsid-inhibitors target the viral capsid and can induce mutations that can be cross-reactive enabling escape from both IVIG and drug. This affects treatment options and should be investigated further.

Introduction

Human enteroviruses (EV) are members of the *Picornaviridae* family classified as species A-D and rhinovirus (HRV) species A-C. They cause a wide variety of disease from mild infections to severe cases of meningitis or myocarditis. These viruses are widespread, with multiple genotypes co-circulating and causing outbreaks with new or more virulent types. Treatment options for severe EV infections are limited. Based on the humoral responsiveness of EV infection, IVIG is often given in severe cases. However, the clinical outcome varies and relies on the presence of the neutralizing immunoglobulins (Igs) within a given batch.[1] There is currently no antiviral drug available to treat EV-infected patients. In the nineties pleconaril was developed to combat rhinoviruses infections [2-5] and it is the only anti-enteroviral drug that reached the phase III clinical trials. Pleconaril is a capsid inhibitor designed to dock within a hydrophobic pocket formed by the capsid proteins VP1, VP3 and VP2. The compound leads to stiffening of the capsid structure, preventing RNA release into the cell.[6] Pleconaril has been used as treatment on a compassionate use basis in neonates and immunodeficient patients with severe EV infections (reviewed by Wildenbeest *et al.*[1]). Results on the clinical outcome varied considerably from complete recovery to fatalities. Data on resistance occurrence and development have only been provided in the pleconaril trials against the common cold.[7-9] Remarkably, the reason for these differences in responses was never investigated by means of typing and testing *in vitro* susceptibility of isolated virus strains. The failure to react of EVs to pleconaril could have been attributed to the drug-induced emergence of resistance, but also to infection with a strain that is naturally or intrinsically resistant to pleconaril, as has been described for Coxsackievirus (CV)B3 and EV71,[10-12] but not for EV-infected patients treated with pleconaril.

Pleconaril is now licensed by Scheringh-Plough (2003) and investigated for the treatment of HRV induced exacerbations of asthma and Chronic Obstructive Pulmonary Disease (COPD) in high-risk patients (www.clinicaltrials.gov; NCT00394914), and for the treatment of neonatal sepsis (www.clinicaltrials.gov; NCT00031512). Its potential use in the treatment of these diseases warrants the necessity to investigate the underlying mechanisms of resistance emergence. Furthermore, understanding the mechanisms underlying resistance to capsid inhibitors is crucial for the development of new and improved EV-drugs.

Previous *in vitro* studies showed that Echovirus (E)11 isolates are among the strains most susceptible to pleconaril.[11] We identified the first pleconaril resistant E11 strain from an immunodeficient patient with chronic meningoencephalitis, who had been treated with intravenous immunoglobulin (IVIG) for over 40 years and only recently with pleconaril. The patient was unresponsive to IVIG or pleconaril.[1] E11 resistance was already observed before the patient had been treated with pleconaril, excluding the resistance to be drug-induced. Here we investigate the underlying mechanisms of this intrinsic resistance.

Results

Pleconaril susceptibility and IVIG titers of the patient strains and clinical isolates

Three pleconaril-resistant E11 strains were identified from one patient who had been treated with IVIG over the course of years, and with pleconaril in 2008.[1] All three strains were resistant to pleconaril and neutralized poorly by IVIG (Table 1). Phylogenetic analysis of the VP1 gene indicated the patient strains to be most closely related to another single Dutch E11 strain isolated in 2007 (E11-NL24/ns/CY/07 (GU393798), Fig. 1) that was characterized as a new genogroup G.[13] Unfortunately, there was no material of the strain E11-NL24/ns/CY/07 left to determine pleconaril susceptibility and IVIG neutralization. For comparison, seven available clinical strains isolated from different patients between 2007 and 2010 (genogroups D5 (n=6) and genogroup C (n=1), Fig. 1) and the prototype E11 strain (E11-PT, genogroup B (Fig. 1)) were collected to investigate pleconaril susceptibility and IVIG neutralization among circulating strains. In all the clinical isolates as well as E11-PT, pleconaril was effective in inhibiting replication, with an IC_{50} of <0.050 µg/ml. The IVIG batch contained high neutralizing titers against the clinical strains but not to E11-PT and the E11 C-strain, which was isolated from a patient travelling to Eritrea (21052365, Table 1). These difference in neutralization capacity of IVIG (Table 1) indicates the genetic difference between M07067754 and the clinical E11 D5 and C strains to reflect antigenically different strains.

Table 1. Antiviral activity of pleconaril against and IVIG end-point titrations of echovirus 11 strains.

Strain	Origin	Genogroup	Pleconaril treatment	IC_{50} (µM)	IVIG titration
M07067754	XLA-patient strain (Sept 2007)	G	-	≥32	16
M08084333	XLA-patient strain (May 2008)	G	+	≥32	16
M09053042	XLA-patient strain (May 2009)	G	+	≥32	16
E11-PT	Prototype	B	na	0.003	8
20751046	clinical isolate 2007	D5	na	0.003	512
20750433	clinical isolate 2007	D5	na	0.042	64
20750473	clinical isolate 2007	D5	na	0.003	32
21051708	clinical isolate 2010	D5	na	0.001	128
21051867	clinical isolate 2010	D5	na	0.003	128
21051929	clinical isolate 2010	D5	na	0.003	64
21052365	clinical isolate 2010	C*	na	0.05	16
CBV3 Nancy	control	-	na	≥32	128
EV71	control	-	na	≥32	512
HRV16	control	-	na	0.001	64
E13	control	-	na	0.032	20
CAV9	control	-	na	0.032	256

IC_{50}; 50% inhibitory concentration, IVIG; intravenous immunoglobuline, na; not applicable.
*Clinical isolate was isolated from a patient travelling from Eritrea.

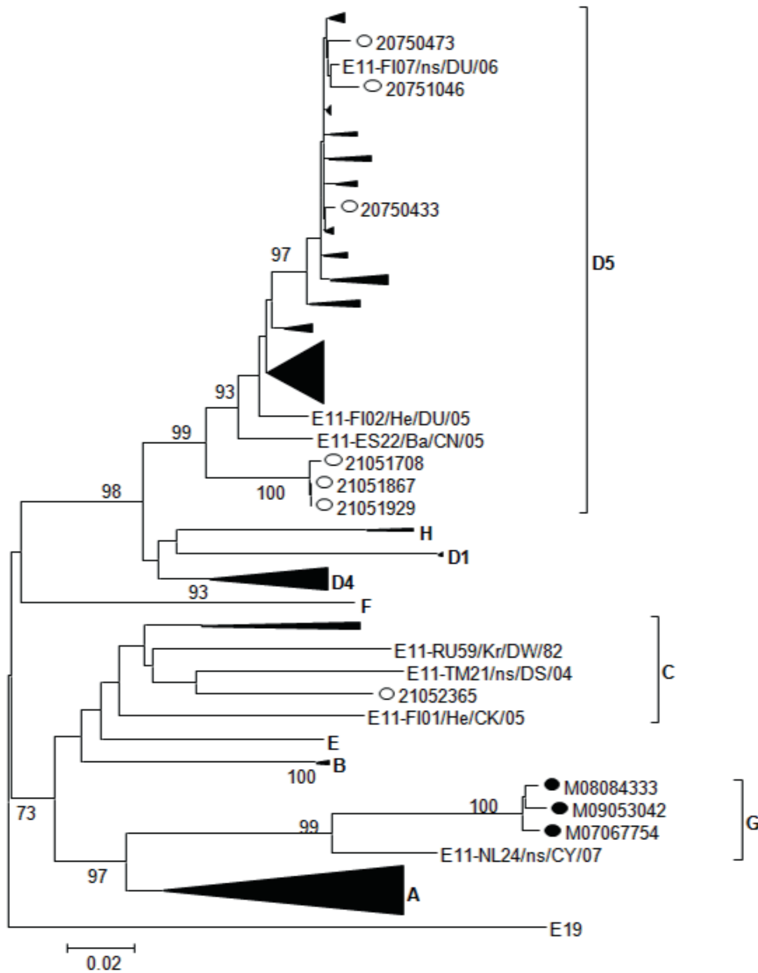


Figure 1. Neighbor-joining tree of MCL-corrected pairwise distances of VP1 sequences of the patient E11 strains. Patient sequences are aligned against VP1 sequences of the E11 Dutch clinical isolates and VP1 sequences of the E11 dataset published by McWilliam Leitch *et al.*[13] Patient strains are indicated in closed circles. Dutch clinical isolates are indicated in open circles. Numbers indicate bootstrap support after 1000 bootstrap replicates. Proportional divergence is indicated by the scale bar.

Amino acid variation of the capsid genes of the E11 pleconaril resistant strains and susceptible clinical E11 strains

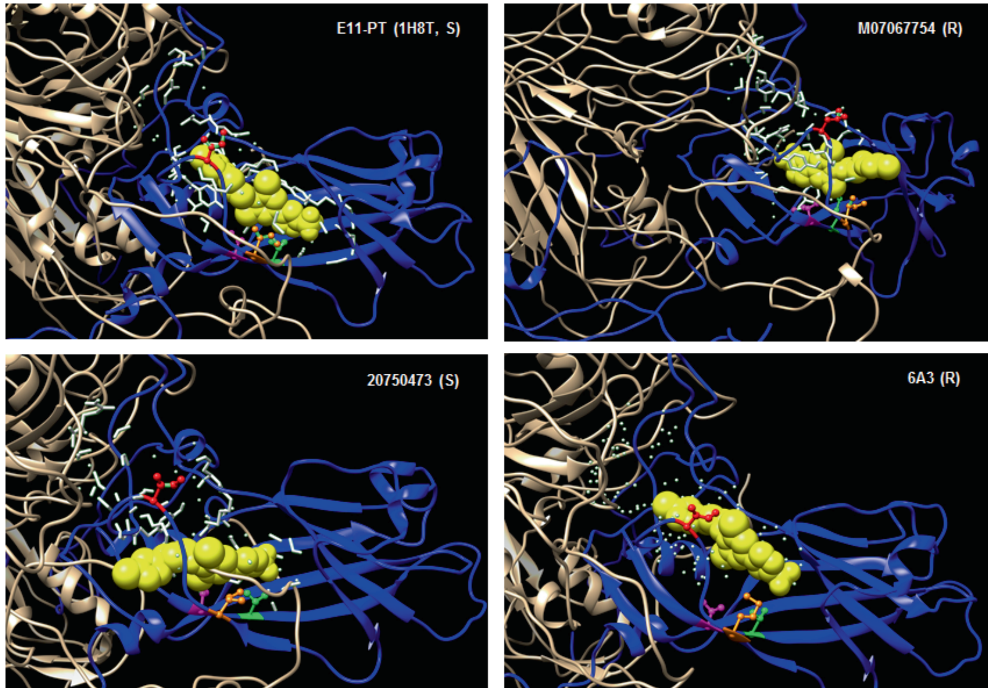
In order to look for resistance markers, genetic analysis of the capsid gene was performed both on the resistant patient strains and sensitive clinical strains. The three patient strains collected over three years (Table 1) displayed only three substitutions within the VP1 gene (Fig. S1, residues 78, 136, 163). In total, 33 residues displayed substitutions specific for the patient strains compared to the clinical and prototype strains (Fig. S1, red boxes). None of the substitutions were identified as culture artefacts, as the similar residues were identified in the original CSF specimens of the patient. Based on the partial VP1 sequence available of

the other genogroup G strain, E11-NL24/ns/CY/07, 5/33 amino acid substitutions were also found in this strain (Fig. S1, residues 64, 82, 91, 117, 128, red boxes). Analysis of the entire P1 region of patient strain M07067754, clinical strain 20750473, and E11-PT (EU443624), indicated a total of 64 amino acid substitutions specific for M07067754 (Fig. S2, red boxes).

Hydrophobic pocket formation and pleconaril docking

Pleconaril docks within a hydrophobic pocket formed within the capsid. Therefore, amino acid substitutions conferring pleconaril resistance are typically located within the pocket. To determine the amino acids involved in pocket formation, 3D-models of the P1 asymmetric unit of the pleconaril-resistant strain M07067754, and the susceptible clinical strain 20750473, were compared with the user-supplied template structure of the prototype strain (PDB 1H8T) (Fig. 2).[14] The amino acids involved in pocket formation of E11-PT, M07067754 and 20750473 are predominantly located in the VP1 gene (Fig. 3 and Fig. S2, black bordered boxes). Both E11-PT and 20750473 display a similar pocket formation (Fig. 2), whereas the pocket formation for M07067754 has been altered and reduced, disabling efficient docking within the pocket of M07067754. PEARL interaction energy values for pleconaril interaction with E11-PT or 20750473 indicated a strong interaction of pleconaril within the pocket in comparison to the interaction of pleconaril within the pocket of M07067754 (Table 2). These interactions are underlined by lower interaction values of the pocket factor in E11-PT or 20750473 indicating that pleconaril binding within the pocket is favored over the pocket factor and therefore outcompetes the pocket factor. In contrast, pleconaril values were lower compared to the pocket factor in M07067754, so pleconaril is predicted not to outcompete the pocket factor in this strain.

Of the 64 M07067754-specific residues, V117I, V119M, and I188L were identified to be involved in formation of the hydrophobic pocket, and thus could confer resistance to pleconaril (Fig. 3). Both V119M and I188L were found to be involved in the structural changes of the pocket, whereas V117I displayed no structural changes in M07067754 compared to the sensitive strains E11-PT or 20750473. Also, the substitution V117I was identified in VP1 sequences published in the GenBank database, including genogroup G strain E11-NL24/ns/CY/07 (Fig. S1). The V119M and I188L substitutions was not found in published sequences. Residue 119 of E11-NL24/ns/CY/07 was similar to clinical and published strains, while data on residue 188 of NL24/ns/CY/07 were unavailable.



	117	119	183	188
E11-PT (S)	V	V	I	I
M07067754 (R)	I	M	I	L
20750473 (S)	V	V	I	I
6A3 (R)	V	I	M	I

Figure 2. Structural modelling of sensitive and resistant E11 strains.
Docking of pleconaril (yellow) within the hydrophobic pocket (grey structure) formed by the capsid proteins VP1 (blue). Amino acid residues 117 (pink), 119 (green), 183 (orange), and 188 (red) lining the pocket are displayed.

Table 2. PEARL interaction values for pleconaril and pocket factor.

Strain	Susceptibility	Pleconaril	Pocket factor
E11-PT	sensitive	-10.81	-6.96
M07067754	resistant	-5.13	-7.03
20750473	sensitive	-9.4	-7.98
6B3	resistant	-2.28	-4.69

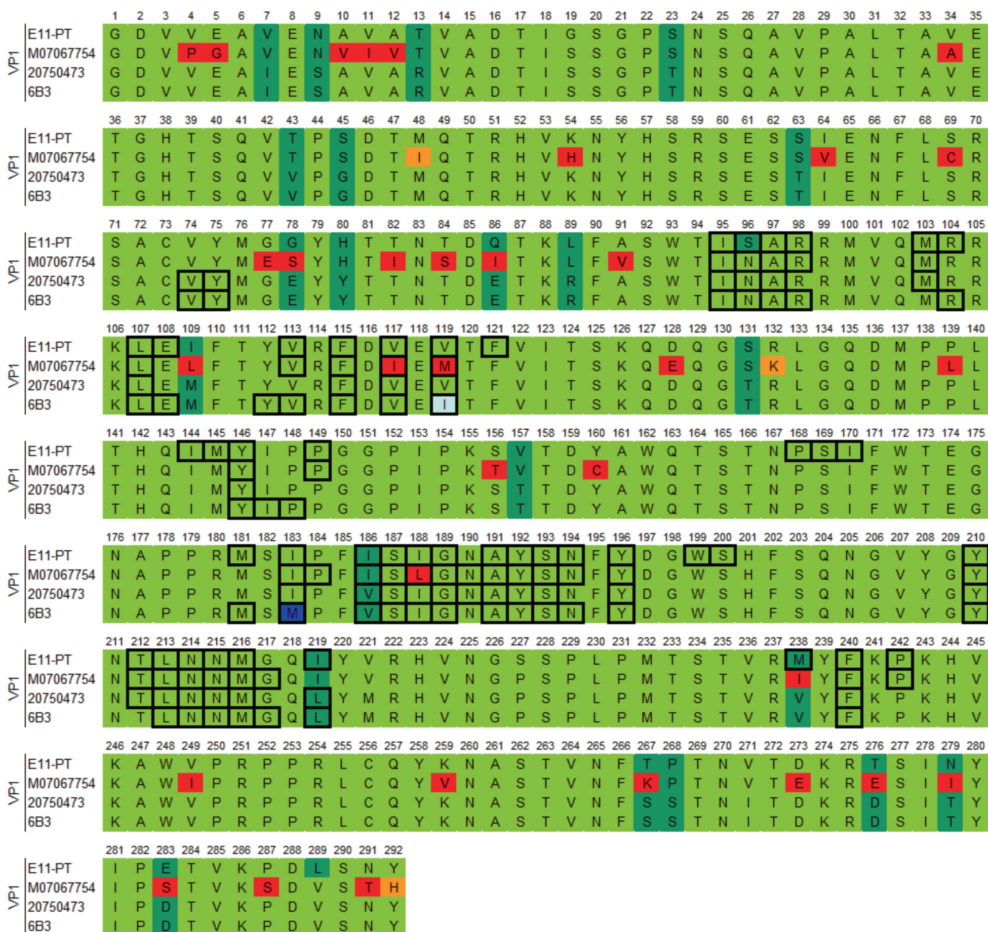


Figure 3. Amino acid alignment of the sensitive prototype (E11-PT) and clinical (20750473) strain with the resistant patient strain (M07067754). Amino acids changes specific for M07067754 are shown in red. Amino acids predicted to line the hydrophobic pocket are shown in black boxes.

Pleconaril induced resistance

To assess whether the amino acid mutations observed *in vivo* can be induced under drug selection, the pleconaril-sensitive clinical strain 20750473 was cultured with increasing concentrations of pleconaril. Resistance to pleconaril was already observed by passage 3 (strain 3A4), displaying an increase in IC_{50} of 1 µmol/L. At passage 4 to 6, the IC_{50} was ≥32 µmol/L, confirming that resistance was induced (Table 3). Modelling of the resistant variant at passage 6 (strain B3 (6B3), Fig. 2) showed a similar pocket formation as shown for the sensitive strains E11-PT and 20750473, yet docking of pleconaril within the pocket was predicted to be inefficient due to the loose structure of the pocket surrounding pleconaril. Similar data were found for the resistant strain B5 at passage 11 (strain 11B5, data not shown). As indicated for the sensitive E11-PT and resistant M07067754 strain, PEARL energy values for pleconaril interaction of the parental strain and strain 6B3 were comparable

confirming the susceptibility difference *in vitro* (Table 2). Comparison of the amino acid sequence showed an I183M substitution after three passages (strain 3A4) and this was retained in further passages (Table 3). Interestingly, site 119 containing the V-M change in the patient strain was also found to be substituted *in vitro* when pleconaril was omitted (V119I). Strain 11B5 containing the V119M substitution was shown to replicate more efficiently than the strain at passage 6 (6B3) (data not shown), indicating a compensatory function of this substitution. IVIG was able to neutralize both the parental sensitive clinical strain and the *in vitro* induced resistant strain 6B3 with titers of 32 (Table 3). In contrast to the suggested structural alteration of the V119M and I188L substitution found in the patient, the I183M substitution was shown to confer resistance through inefficient docking of pleconaril by the protruding sulphate side chain of methionine rather than structurally changing the pocket.

Table 3. Amino acid changes of an *in vitro* induced resistant strain.

Passage	Strain	Pleconaril	IC_{50} (µM)	IVIG titration	Sites VP1			
					<u>117</u>	<u>119</u>	183	<u>188</u>
-	M07067754	-	≥32	16	I	M	I	L
0	20750473	-	0.001	32	V	V	I	I
1	1B4	+	0.1	-	V	V	I	I
2	2F4	+	0.01	-	V	V	I	I
3	3A4	+	1	-	V	V	M	I
4	4A2	+	≥32	-	V	V	M	I
5	5A1	+	≥32	-	V	V	M	I
6	6B3	+	≥32	32	V	V	M	I
7	7A7	-	nd	-	V	I	M	I
8	8B5	-	nd	-	V	I	M	I
9	9B5	-	nd	-	V	I	M	I
10	10D4	-	nd	-	V	I	M	I
11	11B5	-	≥32	32	V	I	M	I

IC_{50}; 50% inhibitory concentration, IVIG; intravenous immunoglobulin, nd; not done.
Underlined are given the sites changed in the patient strain.

Residues lining the hydrophobic pocket confer resistance

To assess the precise contribution of the substitutions predicted to line the hydrophobic pocket of the patient strain (V117I, V119M, and I183L) and the *in vitro* induced pleconaril resistant strains (V119I and I183M), all five mutations were introduced into a sensitive E11 clone (Table 4). Of the seven mutant clones 4 clones produced viable virus, three containing the single mutations V117I, V119M, or I188L and one containing the double mutation V199M and I188L. Clones carrying the V199I and I183M mutations were not viable. The single mutant clones containing the V117I and I188L mutation were found to be sensitive to pleconaril, whereas the single and double mutant clones containing the V119M mutation

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were resistant. Although the I188L substitution is indicated to reflect structural changes, the single mutant containing the I188L substitution does not confer resistance on its own. As such, resistance in the double mutant is primarily conferred by the V119M mutation. IVIG was able to neutralize all four clones with comparable neutralizing titers (Table 4).
The V119I clones were not viable to confirm the compensatory function of this mutation *in vitro*.

Table 4. IC_{50} and IVIG titration of E-11 clones containing substitutions found in M07067754 and 20750473 *in vitro* resistant variants.

	Amino acid changes					Viability	IC_{50} (µM)	IVIG titration
	M07067754			20750473				
	V117I	V119M	I188L	V199I	I183M			
WT						++	0.1	32
1	x					++	0.32	64
2		x				++	≥32	64
3			x			++	0.32	32
4		x	x			+	≥32	32
5	x		x			-	na	na
6	x	x				-	na	na
7	x	x	x			-	na	na
8				x		-	na	na
9					x	-	na	na
10				x	x	-	na	na

IC_{50}; 50% inhibitory concentration, IVIG; intravenous immunoglobulin, na; not applicable.

Discussion

Currently, pleconaril is being re-investigated for the treatment of HRV induced exacerbations of asthma and COPD in high-risk patients (www.clinicaltrials.gov; NCT00394914), and neonatal sepsis (www.clinicaltrials.gov; NCT00031512). It is therefore important to understand the underlying mechanisms of susceptibility and emergence of resistance, in particular of clinical isolates. Our study investigates the genetic and molecular structural basis for pleconaril resistance in the intrinsically susceptible E11 genotype, of which we here describe a resistant strain isolated from a patient before pleconaril treatment was started.
The basis for resistance of pleconaril involves amino acids lining the hydrophobic pocket.[15] In concordance with the phenotypic susceptibility, structural modelling and interaction predictions depicted inefficient docking of pleconaril for the pleconaril resistant patient strain M07067754, while pleconaril was shown to efficiently dock within the pockets of the susceptible prototype and clinical strains. Sequence analysis of the capsid region revealed

three sites that are involved in pocket formation within the capsid genes to have been substituted in the resistant strain; V117I, V119M, and I188L. Both V119M and I188L were found to affect the structure of the pocket. However, by reverse genetics, V119M was shown to confer pleconaril resistance, whereas I188L did not confer resistance on its own.

Notably, in both the patient and the *in vitro* induced resistant strain, resistance was conferred by a methionine residue. However, structural modelling suggests the I183M change to underlie resistance by inefficient docking of pleconaril due to the protruding sulphate-methyl chain of methionine, rather than to structural changes inside the pocket. As the pleconaril resistance *in vivo* was not pleconaril induced and clearly acts by a different mechanism than that found *in vitro*, it is clear that other factors underlie the intrinsic resistance found *in vivo*. Taking into account the immunological divergence of the patient strain, the V119M substitution could have been immunologically induced. The V119M substitution accounts for one of the multiple changes required for immunological divergence, as the clone carrying the V119M substitution alone does not indicate alteration in IVIG susceptibility. Unfortunately, we did not have access to additional historical strains of the patient to determine whether the patient was already infected with this antigenically divergent strain or whether resistance developed under immune selection pressure by the long-term duration of IVIG treatment. Nonetheless, as the other genogroup G strain, E11-NL24/ns/CY/07 did not carry the resistant V119M substitution, we suspect this substitution to have been acquired during replication under IVIG treatment within the patient. Additional studies are required to investigate the role of immunological divergence in affecting drug susceptibility.

In summary, this study indicates that different mechanisms may underlie resistance occurring against a capsid inhibitor in circulating patient strains or induced *in vitro*. This is crucial to take into account when developing new drugs. Our data indicate the importance to investigate susceptibility differences against potential anti-picornaviral drugs not only in laboratory strains but also in the most common circulating EV and HRV genotypes, which are antigenically different from their prototype laboratory strains. Therefore, when EV drugs become available to the clinic, close monitoring of the viral strains is required, especially in immunocompromised individuals with IVIG treatment. Both IVIG and capsid-inhibitors target the viral capsid and can induce mutations that can be cross-reactive enabling escape from both IVIG and drug. This affects treatment options and should be investigated further.

Methods

Viruses

The virus strains isolated from the patient were obtained in 2007 (M07067754), 2008 (M08084333) and 2009 (M09053042).[1] The original CSF samples were stored at the LUMC, Leiden, the Netherlands. The E11 prototype strain, (E11-PT) was obtained from the National

Institute for Public Health and the Environment (RIVM, Bilthoven). Additional clinical E11 strains were isolated in 2007 and 2010 from stool samples sent to the Laboratory of Clinical Virology, AMC, Amsterdam, the Netherlands (Table 1).[16] As control viruses in the pleconaril inhibition assays and IVIG neutralization, E13,[1] CVA9, CVB3, EV71 (RIVM), and HRV16 (a gift of Dr. K. van der Sluijs, Laboratory of Medical Immunology, AMC, Amsterdam, the Netherlands) were used. The E11 clone was a kind gift from Dr. P. Susi (University of Turku, Turku, Finland).

Pleconaril inhibition assay and virus neutralization

The pleconaril inhibition assay and IVIG neutralization were performed as previously described.[1] Pleconaril susceptibility was expressed as the 50% inhibitory concentration (IC_{50}).[1,11] Neutralizing antibody titers within IVIG were calculated by end-point neutralization.

RNA isolation and 5'UTR based real time PCR

All isolates (20 µl) and clinical samples (200 µl) were extracted using the the MagnaPure LC instrument (Roche Diagnostics). RNA was eluted in 50 µl elution buffer and reverse transcribed and detected by EV real-time PCR as previously described.[16-18]

RT-PCR of VP1 and the complete capsid gene

The culture isolates and clinical samples were VP1-genotyped using the semi-nested EV-B-CSF assay by McWilliam Leitch *et al.* [19,20] The VP1 sequences of the E11 clinical strains (KJ830682-KJ830691) were compared to VP1 sequences of the E11 dataset published by McWilliam Leitch *et al.*[21] Sequences were aligned using ClustalW implemented in SSE.[22] Bootstrapped (1000 replicates) maximum likelihood trees were generated using MEGA5 [23] with pairwise deletion for missing data and a gamma distribution value of 0.8.[13]
The complete capsid P1 sequence was amplified by generating overlapping regions (primers are available upon request). The complete capsid region (KJ830692-KJ830693) was aligned using SSE.[22]

Site-directed mutagenesis

Seven mutant E11 clones were constructed by site-directed mutagenesis (QuikChange II XL, Agilent Technologies). The clones contained either single, double or triple mutations at residues 117, 119 and 188 (Table 3). The clones were linearized by digestion with XhoI and RNA was transcribed with T7 RNA polymerase (MEGAscript T7 kit, Ambion). The RNA was transfected into Vero cells using the X-treme GENE transfection reagent (Roche Applied Science). The supernatant was titrated by the TCID50-reed Muench method [24] and sequenced for verification.

In vitro isolation of drug-resistant mutants

A pleconaril sensitive E11 clinical strain (20750473, 100 TCID50/50 µl) was incubated in 10-fold dilutions of pleconaril (100 to 0.0001 µg/ml) in duplo in EMEM-2% FCS for a maximum of 7 days. The virus growing at the maximum pleconaril concentration was passaged further with pleconaril in 10-fold dilutions (100 to 0.0001 µg/ml) in duplo in EMEM-2% FCS. This process was repeated for 6 passages, after which the virus was passaged further 5 times without pleconaril. Of each passage, the viral isolates were stored at -20 ^{o}C for VP1 sequencing and *in vitro* susceptibility testing.

In silico modelling of the VP1-4 complex encoded by the P1 gene

Amino acid sequences of VP1-4 of E11-PT strain were submitted to i_TASSER [25] providing PDB entry 1H8T [14] as user-supplied template structure for combined *ab initio* and homology modeling. Top 3D-models of VP1-4 (judged by TASSER C-scores and structure similarity with Echovirus VPs assayed by MATRAS)[26] were projected on the known structure of Echovirus VP1-4 complex to create a 3D-model of the asymmetric unit of the EV capsid. Steric clashes were removed via CHIRON.[27] The 3D-structure of pleconaril was taken from PDBid 1C8M. Docking of pleconaril was done by PATCHDOCK.[28] The PATCHDOCK top 20 results were ranked according to their energy profiles determined by means of PEARLS in order to select the most stable complex.[29] Pockets were identified by POCKETFINDER.[30] RASTOP, CHIMERA [31] and the PDBsum facility of PROFUNC [32] were used to display the figures.

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Group	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
resistant patient strains	M07067754	G	D	V	P	G	A	V	E	N	V	I	V	T	V	A	D	T	I	S	S	G	P	S	N	S	Q	A	V	P	A	L	T	A	A	E
	M08084333	G	D	V	P	G	A	I	E	N	V	I	V	T	V	A	D	T	I	S	S	G	P	S	N	S	Q	A	V	P	A	L	T	A	A	E
	M09053042	G	D	V	P	G	A	V	E	N	V	I	V	T	V	A	D	T	I	S	S	G	P	S	N	S	Q	A	V	P	A	L	T	A	A	E
sensitive E11 strains	E11-PT	G	D	V	V	E	A	V	E	N	A	V	A	T	V	A	D	T	I	G	S	G	P	S	N	S	Q	A	V	P	A	L	T	A	V	E
	20750433	G	D	V	V	E	A	I	E	S	A	V	A	R	V	A	D	T	I	S	S	G	P	T	N	S	Q	A	V	P	A	L	T	A	V	E
	20750473	G	D	V	V	E	A	I	E	S	A	V	A	R	V	A	D	T	I	S	S	G	P	T	N	S	Q	A	V	P	A	L	T	A	V	E
	20751046	G	D	V	V	E	A	I	E	S	A	V	A	R	V	A	D	T	I	S	S	G	P	T	N	S	Q	A	V	P	A	L	T	A	V	E
	21051708	G	D	V	V	E	A	I	E	S	A	V	A	R	V	A	D	T	I	S	S	G	P	T	N	S	Q	A	V	P	A	L	T	A	V	E
	21051867	G	D	V	V	E	A	I	E	S	A	V	A	R	V	A	D	T	I	S	S	G	P	T	N	S	Q	A	V	P	A	L	T	A	V	E
	21051929	G	D	V	V	E	A	I	E	S	A	V	A	R	V	A	D	T	I	S	S	G	P	T	N	S	Q	A	V	P	A	L	T	A	V	E
	21052365	G	D	V	V	E	A	V	E	N	A	M	A	R	V	A	D	T	I	G	S	G	P	S	N	S	Q	A	V	P	A	L	T	A	V	E
	E11-NL24/ns/CY/07	G	D	V	V	R	A	R	G	K	S	L	A	R	V	A	D	H	I	G	S	G	P	S	N	S	Q	A	V	H	A	L	T	A	V	E

Group	Strain	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
resistant patient strains	M07067754	T	G	H	T	S	Q	V	T	P	S	D	T	I	Q	T	R	H	V	H	N	Y	H	S	R	S	E	S	T	V	E	N	F	L	C	R
	M08084333	T	G	H	T	S	Q	V	T	P	S	D	T	I	Q	T	R	H	V	H	N	Y	H	S	R	S	E	S	T	V	E	N	F	L	C	R
	M09053042	T	G	H	T	S	Q	V	T	P	S	D	T	I	Q	T	R	H	V	H	N	Y	H	S	R	S	E	S	T	V	E	N	F	L	C	R
sensitive E11 strains	E11-PT	T	G	H	T	S	Q	V	T	P	S	D	T	M	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	T	I	E	N	F	L	S	R
	20750433	T	G	H	T	S	Q	V	V	P	G	D	T	M	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	T	I	E	N	F	L	S	R
	20750473	T	G	H	T	S	Q	V	V	P	G	D	T	M	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	T	I	E	N	F	L	S	R
	20751046	T	G	H	T	S	Q	V	V	P	G	D	T	M	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	T	I	E	N	F	L	S	R
	21051708	T	G	H	T	S	Q	V	V	P	G	D	T	M	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	T	I	E	N	F	L	S	R
	21051867	T	G	H	T	S	Q	V	V	P	G	D	T	M	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	T	I	E	N	F	L	S	R
	21051929	T	G	H	T	S	Q	V	V	P	G	D	T	M	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	T	I	E	N	F	L	S	R
	21052365	T	G	H	T	S	Q	V	T	P	S	D	T	I	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	T	I	E	N	F	L	S	R
	E11-NL24/ns/CY/07	T	G	H	T	S	Q	V	V	P	G	D	T	I	Q	T	R	H	V	K	N	Y	H	S	R	S	E	S	S	V	E	N	F	L	S	R

Group	Strain	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
resistant patient strains	M07067754	S	A	C	V	Y	M	E	S	Y	H	T	I	N	S	D	I	T	K	L	F	V	S	W	T	I	N	A	R	R	M	V	Q	M	R	R
	M08084333	S	A	C	V	Y	M	E	S	Y	H	T	I	N	S	D	I	T	K	L	F	V	S	W	T	I	N	A	R	R	M	V	Q	M	R	R
	M09053042	S	A	C	V	Y	M	E	T	Y	H	T	I	N	S	D	I	T	K	L	F	V	S	W	T	I	N	A	R	R	M	V	Q	M	R	R
sensitive E11 strains	E11-PT	S	A	C	V	Y	M	G	G	Y	H	T	T	N	T	D	Q	T	K	L	F	A	S	W	T	I	S	A	R	R	M	V	Q	M	R	R
	20750433	S	A	C	V	Y	M	G	E	Y	Y	T	T	N	T	D	E	T	K	R	F	A	S	W	T	I	N	A	R	R	M	V	Q	M	R	R
	20750473	S	A	C	V	Y	M	G	E	Y	Y	T	T	N	T	D	E	T	K	R	F	A	S	W	T	I	N	A	R	R	M	V	Q	M	R	R
	20751046	S	A	C	V	Y	M	G	E	Y	Y	T	T	N	T	D	E	T	K	R	F	A	S	W	T	I	N	A	R	R	M	V	Q	M	R	R
	21051708	S	A	C	V	Y	M	G	E	Y	Y	T	T	N	T	D	E	T	K	R	F	A	N	W	T	I	S	A	R	R	M	V	Q	M	R	R
	21051867	S	A	C	V	Y	M	G	E	Y	Y	T	T	N	T	D	E	T	K	R	F	A	N	W	T	I	S	A	R	R	M	V	Q	M	R	R
	21051929	S	A	C	V	Y	M	G	E	Y	Y	T	T	N	T	D	E	T	K	R	F	A	N	W	T	I	S	A	R	R	M	V	Q	M	R	R
	21052365	S	A	C	V	Y	M	G	E	Y	H	T	T	N	T	D	Q	T	K	L	F	A	S	W	T	I	S	A	R	R	M	V	Q	M	R	R
	E11-NL24/ns/CY/07	S	A	C	V	Y	M	G	R	Y	Y	T	I	N	T	D	V	T	K	R	F	V	S	W	T	I	N	A	R	R	M	V	Q	M	R	S

Group	Strain	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
resistant patient strains	M07067754	K	L	E	L	F	T	Y	V	R	F	D	I	E	M	T	F	V	I	T	S	K	Q	E	Q	G	S	K	L	G	Q	D	M	P	L	L
	M08084333	K	L	E	L	F	T	Y	V	R	F	D	I	E	M	T	F	V	I	T	S	K	Q	E	Q	G	S	K	L	G	Q	D	M	P	L	L
	M09053042	K	L	E	L	F	T	Y	V	R	F	D	I	E	M	T	F	V	I	T	S	K	Q	E	Q	G	S	K	L	G	Q	V	M	P	L	L
sensitive E11 strains	E11-PT	K	L	E	I	F	T	Y	V	R	F	D	V	E	V	T	F	V	I	T	S	K	Q	D	Q	G	S	R	L	G	Q	D	M	P	P	L
	20750433	K	L	E	M	F	T	Y	V	R	F	D	V	E	V	T	F	V	I	T	S	K	Q	D	Q	G	T	K	L	G	Q	D	M	P	P	L
	20750473	K	L	E	M	F	T	Y	V	R	F	D	V	E	V	T	F	V	I	T	S	K	Q	D	Q	G	T	Q	L	G	Q	D	M	P	P	L
	20751046	K	L	E	M	F	T	Y	V	R	F	D	V	E	V	T	F	V	I	T	S	K	Q	D	Q	G	T	Q	L	G	Q	D	M	P	P	L
	21051708	K	L	E	M	F	T	Y	V	R	F	D	V	E	V	T	F	V	I	T	S	K	Q	D	Q	G	T	Q	L	G	Q	D	M	P	P	L
	21051867	K	L	E	M	F	T	Y	V	R	F	D	V	E	V	T	F	V	I	T	S	K	Q	D	Q	G	T	Q	L	G	Q	D	M	P	P	L
	21051929	K	L	E	M	F	T	Y	V	R	F	D	V	E	V	T	F	V	I	T	S	K	Q	D	Q	G	T	Q	L	G	Q	D	M	P	P	L
	21052365	K	L	E	I	F	T	Y	V	R	F	D	V	E	V	T	F	V	I	T	S	K	Q	D	Q	G	S	Q	L	G	Q	D	M	P	P	L
	E11-NL24/ns/CY/07	K	L	E	M	F	T	Y	V	R	F	D	I	E	V	T	F	V	I	T	S	*	Q	E	Q	G	T	K	L	G	Q	D	M	P	P	L

Group	Strain	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175
resistant patient strains	M07067754	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	T	V	T	D	C	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	M08084333	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	T	V	T	D	C	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	M09053042	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	T	V	T	D	C	A	W	K	T	S	T	N	P	S	I	F	W	T	E	G
sensitive E11 strains	E11-PT	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	S	V	T	D	Y	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	20750433	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	S	T	T	D	Y	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	20750473	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	S	T	T	D	Y	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	20751046	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	S	T	T	D	Y	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	21051708	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	S	T	T	D	Y	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	21051867	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	S	T	T	D	Y	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	21051929	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	S	T	T	D	Y	A	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	21052365	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	S	V	T	D	Y	T	W	Q	T	S	T	N	P	S	I	F	W	T	E	G
	E11-NL24/ns/CY/07	T	H	Q	I	M	Y	I	P	P	G	G	P	I	P	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Group	Strain	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210
resistant patient strains	M07067754	N	A	P	P	R	M	S	I	P	F	I	S	L	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	M08084333	N	A	P	P	R	M	S	I	P	F	I	S	L	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	M09053042	N	A	P	P	R	M	S	I	P	F	I	S	L	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
sensitive E11 strains	E11-PT	N	A	P	P	R	M	S	I	P	F	I	S	I	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	20750433	N	A	P	P	R	M	S	I	P	F	V	S	I	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	20750473	N	A	P	P	R	M	S	I	P	F	V	S	I	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	20751046	N	A	P	P	R	M	S	I	P	F	V	S	I	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	21051708	N	A	P	P	R	M	S	I	P	F	V	S	I	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	21051867	N	A	P	P	R	M	S	I	P	F	V	S	I	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	21051929	N	A	P	P	R	M	S	I	P	F	V	S	I	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	21052365	N	A	P	P	R	M	S	I	P	F	I	S	I	G	N	A	Y	S	N	F	Y	D	G	W	S	H	F	S	Q	N	G	V	Y	G	Y
	E11-NL24/ns/CY/07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Group	Strain	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245
resistant patient strains	M07067754	N	T	L	N	H	M	G	Q	I	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	I	Y	F	K	P	K	H	V
	M08084333	N	T	L	N	H	M	G	Q	I	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	I	Y	F	K	P	K	H	V
	M09053042	N	T	L	N	H	M	G	Q	I	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	I	Y	F	K	P	K	H	V
sensitive E11 strains	E11-PT	N	T	L	N	H	M	G	Q	I	Y	V	R	H	V	N	G	S	S	P	L	P	M	T	S	T	V	R	M	Y	F	K	P	K	H	V
	20750433	N	T	L	N	N	M	G	Q	L	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	V	Y	F	K	P	K	H	V
	20750473	N	T	L	N	N	M	G	Q	L	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	V	Y	F	K	P	K	H	V
	20751046	N	T	L	N	N	M	G	Q	L	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	V	Y	F	K	P	K	H	V
	21051708	N	T	L	N	N	M	G	Q	L	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	V	Y	F	K	P	K	H	V
	21051867	N	T	L	N	N	M	G	Q	L	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	V	Y	F	K	P	K	H	V
	21051929	N	T	L	N	N	M	G	Q	L	Y	V	R	H	V	N	G	P	S	P	L	P	M	T	S	T	V	R	V	Y	F	K	P	K	H	V
	21052365	N	T	L	N	H	M	G	Q	L	Y	V	R	H	V	N	G	S	S	P	L	P	M	T	S	T	V	R	M	Y	F	K	P	K	H	V
	E11-NL24/ns/CY/07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Group	Strain	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280
resistant patient strains	M07067754	K	A	W	I	P	R	P	P	R	L	C	Q	Y	V	N	A	S	T	V	N	F	K	P	T	N	V	T	E	K	R	E	S	I	I	Y
	M08084333	K	A	W	I	P	R	P	P	R	L	C	Q	Y	V	N	A	S	T	V	N	F	K	P	T	N	V	T	E	K	R	E	S	I	I	Y
	M09053042	K	A	W	I	P	R	P	P	R	L	C	Q	Y	V	N	A	S	T	V	N	F	K	P	T	N	V	T	E	K	R	E	S	I	I	Y
sensitive E11 strains	E11-PT	K	A	W	V	P	R	P	P	R	L	C	Q	Y	K	N	A	S	T	V	N	F	T	P	T	N	V	T	D	K	R	T	S	I	N	Y
	20750433	K	A	W	V	P	R	P	P	R	L	C	Q	Y	K	N	A	S	T	V	N	F	S	P	T	N	V	T	D	K	R	D	S	I	T	Y
	20750473	K	A	W	V	P	R	P	P	R	L	C	Q	Y	K	N	A	S	T	V	N	F	S	S	T	N	V	T	D	K	R	D	S	I	T	Y
	20751046	K	A	W	V	P	R	P	P	R	L	C	Q	Y	K	N	A	S	T	V	N	F	S	S	T	N	V	T	D	K	R	D	S	I	T	Y
	21051708	K	A	W	V	P	R	P	P	R	L	C	Q	Y	K	N	A	S	T	V	N	F	S	S	T	N	V	T	D	K	R	D	S	I	T	Y
	21051867	K	A	W	V	P	R	P	P	R	L	C	Q	Y	K	N	A	S	T	V	N	F	S	S	T	N	V	T	D	K	R	D	S	I	T	Y
	21051929	K	A	W	V	P	R	P	P	R	L	C	Q	Y	K	N	A	S	T	V	N	F	S	S	T	N	V	T	D	K	R	D	S	I	T	Y
	21052365	K	A	W	V	P	R	P	P	R	L	C	Q	Y	K	N	A	S	T	V	N	F	T	P	T	N	V	T	S	K	R	A	S	I	T	Y
	E11-NL24/ns/CY/07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Group	Strain	281	282	283	284	285	286	287	288	289	290	291	292
resistant patient strains	M07067754	I	P	S	T	V	K	S	D	V	S	T	H
	M08084333	I	P	S	T	V	K	S	D	V	S	T	H
	M09053042	I	P	S	T	V	K	S	D	V	S	T	H
sensitive E11 strains	E11-PT	I	P	E	T	V	K	P	D	L	S	N	Y
	20750433	I	P	D	T	V	K	P	D	V	S	N	Y
	20750473	I	P	D	T	V	K	P	D	V	S	N	Y
	20751046	I	P	D	T	V	K	P	D	V	S	N	Y
	21051708	I	P	D	T	V	K	P	D	V	S	N	H
	21051867	I	P	D	T	V	K	P	D	V	S	N	H
	21051929	I	P	D	T	V	K	P	D	V	S	N	H
	21052365	I	P	D	T	V	K	P	D	V	S	N	Y
	E11-NL24/ns/CY/07	-	-	-	-	-	-	-	-	-	-	-	-

Figure S1. Amino acid alignment of the VP1 region of the resistant patient E11 strains against sensitive strains and E11-NL24/ns/CY/07. Conserved regions are depicted in light green, whereas non-conserved amino acids identified in the both resistant and sensitive strains are depicted in dark green. Amino acids changes specific for M07067754 and identified in E11-NL24/ns/CY/07are shown in red. Amino acids changes specific for E11-NL24/ns/CY/07 are shown in yellow. The region for E11-NL24/ns/CY/07 not sequenced is indicated in grey.

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Protein	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
VP4	E11-PT	M	G	A	Q	V	S	T	Q	K	T	G	A	H	E	T	G	L	N	A	S	G	S	S	I	I	H	Y	T	N	I	N	Y	Y	K	D
	M07067754	M	G	A	Q	V	S	T	Q	K	T	G	A	H	E	T	G	L	S	A	S	G	N	S	I	I	H	Y	T	N	I	N	Y	Y	K	D
	20750473	M	G	A	Q	V	S	T	Q	K	T	G	A	H	E	T	G	L	N	A	S	G	N	S	I	I	H	Y	T	N	I	N	Y	Y	K	D
	6B3	M	G	A	Q	V	S	T	Q	K	T	G	A	H	E	T	G	L	N	A	S	G	N	S	I	I	H	Y	T	N	I	N	Y	Y	K	D

Protein	Strain	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
VP4	E11-PT	A	A	S	N	S	A	N	R	Q	E	F	S	Q	D	P	G	K	F	T	E	P	V	K	D	I	M	V	K	S	L	P	A	L	N
	M07067754	A	A	S	N	S	A	N	R	Q	D	F	S	Q	D	P	G	K	F	T	E	P	V	K	D	I	M	A	K	S	L	P	A	L	N
	20750473	A	A	S	N	S	A	N	R	Q	D	F	T	Q	D	P	G	K	F	T	E	P	V	K	D	I	M	I	K	S	M	P	A	L	N
	6B3	A	A	S	N	S	A	N	R	Q	D	F	T	Q	D	P	G	K	F	T	E	P	V	K	D	I	M	I	K	S	M	P	A	L	N

Protein	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
VP2	E11-PT	S	P	S	A	E	E	C	G	Y	S	D	R	V	R	S	I	T	L	G	N	S	T	I	T	T	Q	E	S	A	N	V	V	V	G	Y
	M07067754	S	P	S	A	E	E	C	G	Y	S	D	R	V	R	S	I	T	L	G	N	S	T	I	T	T	Q	E	S	A	N	V	V	V	A	Y
	20750473	S	P	S	A	E	E	C	G	Y	S	D	R	V	R	S	I	T	L	G	N	S	T	I	T	T	Q	E	C	A	N	V	V	V	A	Y
	6B3	S	P	S	A	E	E	C	G	Y	S	D	R	V	R	S	I	T	L	G	N	S	T	I	T	T	Q	E	C	A	N	V	V	V	A	Y

Protein	Strain	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
VP2	E11-PT	G	R	W	P	E	Y	L	K	D	N	E	A	T	A	E	D	Q	P	T	Q	P	D	V	A	T	C	R	F	Y	T	L	E	S	V	T
	M07067754	G	R	W	P	N	Y	L	K	D	D	E	A	T	A	E	D	Q	P	T	Q	P	D	V	A	T	C	R	F	F	T	L	E	S	V	T
	20750473	G	R	W	P	E	Y	L	S	D	K	E	A	T	A	E	D	Q	P	T	Q	P	D	V	A	T	C	R	F	Y	T	L	E	S	V	T
	6B3	G	R	W	P	E	Y	L	S	D	K	E	A	T	A	E	D	Q	P	T	Q	P	D	V	A	T	C	R	F	Y	T	L	E	S	V	T

Protein	Strain	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
VP2	E11-PT	W	E	R	D	S	P	G	W	W	W	K	F	P	D	A	L	K	D	M	G	L	F	G	Q	N	M	Y	Y	H	Y	L	G	R	A	G
	M07067754	W	E	K	G	S	P	G	W	W	W	K	F	P	D	A	L	K	D	M	G	L	F	G	Q	N	M	Y	Y	H	Y	L	G	R	A	G
	20750473	W	E	R	D	S	P	G	W	W	W	K	F	P	D	A	L	K	D	M	G	L	F	G	Q	N	M	Y	Y	H	Y	L	G	R	A	G
	6B3	W	E	R	D	S	P	G	W	W	W	K	F	P	D	A	L	K	D	M	G	L	F	G	Q	N	M	Y	Y	H	Y	L	G	R	A	G

Protein	Strain	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
VP2	E11-PT	Y	T	L	H	V	Q	C	N	A	S	K	F	H	Q	G	C	L	L	V	V	C	V	P	E	A	E	M	G	C	S	Q	V	D	G	T
	M07067754	Y	T	I	H	V	Q	C	N	A	S	K	F	H	Q	G	C	L	L	V	V	C	V	P	E	A	E	M	G	C	S	Q	I	D	G	T
	20750473	Y	T	I	H	V	Q	C	N	A	S	K	F	H	Q	G	C	L	L	V	V	C	V	P	E	A	E	M	G	C	S	D	V	T	G	V
	6B3	Y	T	I	H	V	Q	C	N	A	S	K	F	H	Q	G	C	L	L	V	V	C	V	P	E	A	E	M	G	C	S	D	V	T	G	V

Protein	Strain	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175
VP2	E11-PT	V	N	E	H	G	L	S	E	G	E	T	A	K	K	F	S	S	T	S	T	N	G	T	N	T	V	Q	T	I	V	T	N	A	G	M
	M07067754	M	N	E	H	S	L	S	E	G	E	T	A	K	K	F	T	N	T	P	T	N	A	A	N	T	V	Q	S	I	V	T	N	A	G	M
	20750473	V	S	E	H	A	I	S	E	G	E	T	A	K	K	F	S	A	T	A	T	N	G	A	H	T	V	Q	S	L	V	T	N	A	G	M
	6B3	V	S	E	H	A	I	S	E	G	E	T	A	K	K	F	S	A	T	A	T	N	G	A	H	T	V	Q	S	L	V	T	N	A	G	M

Protein	Strain	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210
VP2	E11-PT	G	V	G	V	G	N	L	T	I	Y	P	H	Q	W	I	N	L	R	T	N	N	C	A	T	I	V	M	P	Y	I	N	N	V	P	M
	M07067754	G	V	G	V	G	N	L	T	I	F	P	H	Q	W	I	N	L	R	T	N	N	C	A	T	I	V	M	P	Y	I	N	N	V	P	M
	20750473	G	V	G	V	G	N	L	T	I	Y	P	H	Q	W	I	N	L	R	T	N	N	S	A	T	I	V	M	P	Y	I	N	S	V	P	M
	6B3	G	V	G	V	G	N	L	T	I	Y	P	H	Q	W	I	N	L	R	T	N	N	S	A	T	I	V	M	P	Y	I	N	S	V	P	M

Protein	Strain	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245
VP2	E11-PT	D	N	M	F	R	H	H	N	F	T	L	M	I	I	P	F	V	P	L	D	Y	S	S	D	S	S	T	Y	V	P	I	T	V	T	V
	M07067754	D	N	M	F	R	H	H	N	F	T	L	M	I	I	P	F	V	P	L	N	Y	S	P	D	S	S	T	Y	V	P	I	T	V	T	V
	20750473	D	N	M	F	R	H	H	N	F	T	L	M	I	I	P	F	V	S	L	E	Y	S	S	D	A	S	T	Y	V	P	I	T	V	T	V
	6B3	D	N	M	F	R	H	H	N	F	T	L	M	I	I	P	F	V	S	L	E	Y	S	S	D	A	S	T	Y	V	P	I	T	V	T	V

Protein	Strain	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262
VP2	E11-PT	A	P	M	C	A	E	Y	N	G	L	R	L	S	T	S	L	Q
	M07067754	A	P	M	C	A	E	Y	N	G	L	R	L	A	T	S	L	Q
	20750473	A	P	M	C	A	E	Y	N	G	L	R	L	A	T	S	V	Q
	6B3	A	P	M	C	A	E	Y	N	G	L	R	L	A	T	S	V	Q

Protein	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
VP3	E11-PT	G	L	P	V	M	N	T	P	G	S	N	Q	F	L	T	S	D	D	F	Q	S	P	S	A	M	P	Q	F	D	V	T	P	E	L	N
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	20750473	G	L	P	V	M	N	T	P	G	S	N	Q	F	L	T	S	D	D	F	Q	S	P	S	A	M	P	Q	F	D	V	T	P	E	L	D
	6B3	G	L	P	V	M	N	T	P	G	S	N	Q	F	L	T	S	D	D	F	Q	S	P	S	V	M	P	Q	F	D	V	T	P	E	L	D

Protein	Strain	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70
VP3	E11-PT	I	P	G	E	V	Q	N	L	M	E	I	A	E	V	D	S	V	V	P	V	N	N	V	E	G	K	L	D	T	M	E	V	Y	R	I
	M07067754	I	P	G	E	V	R	N	L	M	E	I	A	E	V	D	S	V	V	P	V	N	N	V	K	D	K	L	D	A	M	D	I	Y	Q	I
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	6B3	I	P	G	E	V	K	N	L	M	E	I	A	E	V	D	S	V	V	P	V	N	N	V	K	G	K	L	D	T	M	D	I	F	R	I

Protein	Strain	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105
VP3	E11-PT	P	V	Q	S	G	N	H	Q	S	D	Q	V	F	G	F	Q	V	Q	P	G	L	D	S	V	F	K	H	T	L	L	G	E	I	L	N
	M07067754	P	V	Q	S	G	N	H	Q	S	S	Q	V	F	G	F	Q	V	Q	P	G	L	E	N	V	F	K	H	T	L	L	G	E	I	L	N
	20750473	P	V	Q	S	G	N	H	Q	G	T	Q	V	F	G	F	Q	V	Q	P	G	L	D	S	V	F	K	H	T	L	L	G	E	I	L	N
	6B3	P	V	Q	S	G	N	H	Q	G	T	Q	V	F	G	F	Q	V	Q	P	G	L	D	S	V	F	K	H	T	L	L	G	E	I	L	N

Protein	Strain	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
VP3	E11-PT	Y	F	A	H	W	S	G	S	I	K	L	T	F	V	F	C	G	S	A	M	A	T	G	K	F	L	L	A	Y	A	P	P	G	A	N
	M07067754	Y	Y	A	H	W	S	G	S	I	K	L	T	F	I	F	C	G	S	A	M	A	T	G	K	F	L	L	A	Y	A	P	P	G	A	N
	20750473	Y	Y	A	H	W	S	G	S	V	K	L	T	F	V	F	C	G	S	A	M	A	T	G	K	F	L	L	A	Y	S	P	P	G	A	N
	6B3	Y	Y	A	H	W	S	G	S	V	K	L	T	F	V	F	C	G	S	A	M	A	T	G	K	F	L	L	A	Y	S	P	P	G	A	N

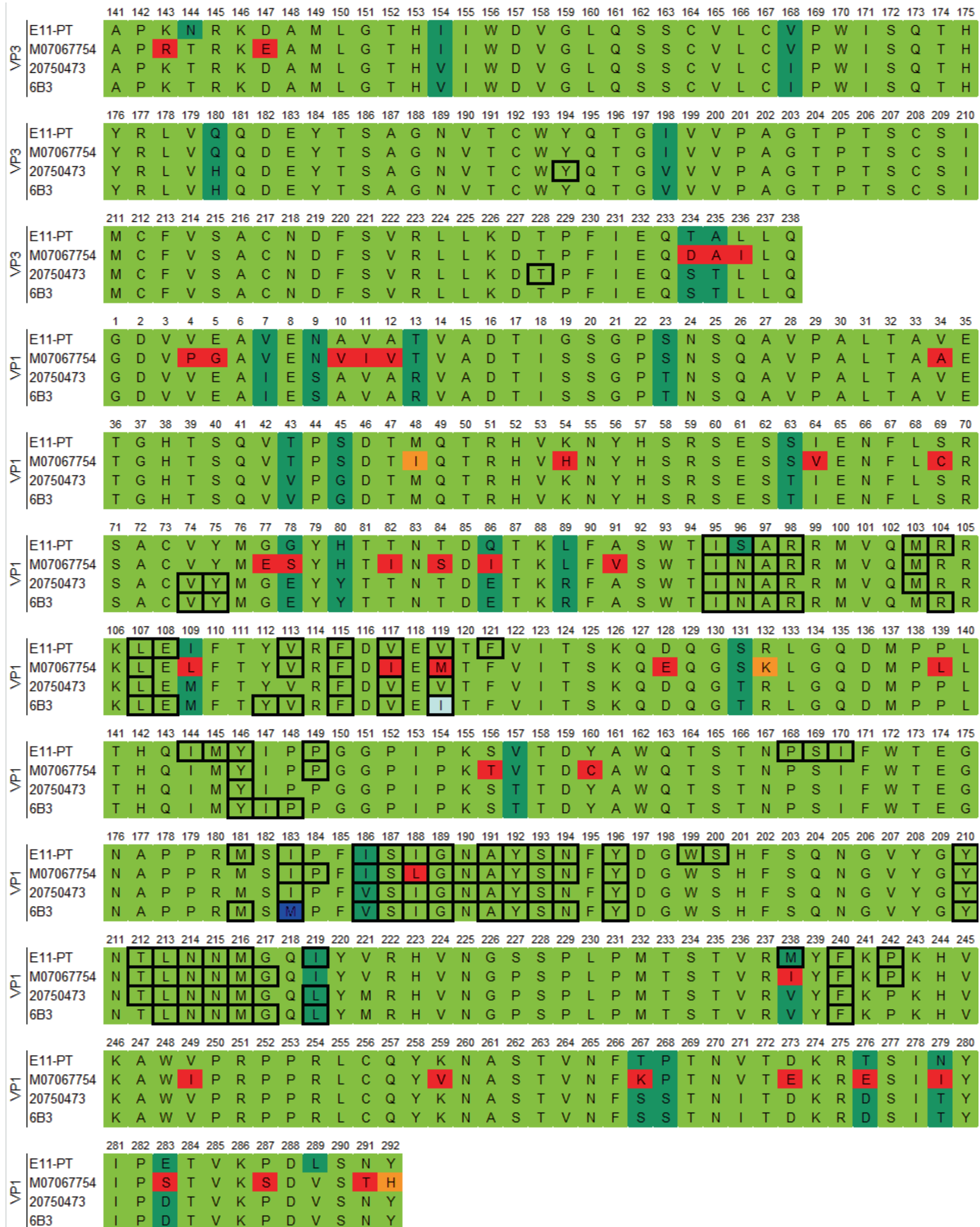

Figure S2. Amino acid alignment of complete capsid of the sensitive and resistant E11 strains. Conserved regions identified among the sensitive prototype (E11-PT), the resistant patient strain (M07067754), the sensitive clinical (20750473) strain and the *in vitro* induced resistant strain (6B3) are depicted in light green, whereas non-conserved amino acids identified in the both resistant and sensitive strains are depicted in dark green. Amino acids changes specific for M07067754 are shown in red. Amino acids changes specific for 6B3 are shown in dark blue. Residue 119 in strain 6B3 is a valine, but is depicted as isoleucine (additional substitution identified in strain 11B5) in light blue for reference. Amino acids predicted to line the hydrophobic pocket are shown in black boxes.

Chapter 9

Successful IVIG treatment of human parechovirus-associated dilated cardiomyopathy in an infant

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Abstract

Human parechoviruses (HPeVs) are closely related to human enteroviruses and exhibit many similarities in disease spectrum and symptoms. HPeV1 is most commonly associated with mild disease, but rare associations with severe disease such as myocarditis have been reported. Currently, no treatment is available for severe HPeV infections.

In this case report we describe an infant with a severe dilated cardiomyopathy in whom HPeV1 was revealed to be the only identifiable cause. The infant was treated with intravenous immunoglobulins (IVIGs) and recovered completely. *In vivo* blood samples revealed a high HPeV1 antibody titer after treatment with IVIGs. *In vitro* IVIGs contained high titers of neutralizing antibodies against HPeV1.

Our hypothesis is that patients with myocarditis caused by viruses with a high prevalence in the population and hence high antibody titers in IVIGs are likely to benefit from treatment with IVIGs. More research, combining virological and clinical data, is needed to see whether this hypothesis is true.

Introduction

Human parechoviruses (HPeVs) are closely related to human enteroviruses (HEVs) and both belong to the *Picornaviridae* family. HPeVs exhibit many similarities to HEVs in symptoms and spectrum of disease.[1,2] HPeV1 and HPeV3 are the most common types of the currently known 16 subtypes, mainly infecting young children under the age of 2 years.[1,3,4] HPeV3 is associated with meningitis and sepsis-like illness especially in neonates and children aged <6 months,[5-8] whereas HPeV1 usually causes mild gastrointestinal and respiratory disease, although meningitis and sepsis-like illness may occur.[5] As incidentally reported in case reports and small studies, HPeV1 has been associated with myocarditis,[9,10] encephalitis,[11] encephalomyelitis,[12] acute flaccid paralysis,[13] sudden infant death,[14] and necrotising enterocolitis.[15] The prevalence of HPeV1 is high in young children.[3,4,16] As was revealed in a longitudinal study, most children become seropositive before the age of 2 years.[17] In adults, 95 to 99% have antibodies against HPeV1.[17] Symptomatic HPeV infection in adults is rarely reported. Currently there is no treatment available for severe infections with HPeV (reviewed in Tauriainen *et al.*[18]). In neonates with severe HEV infections intravenous immunoglobulins (IVIGs) is randomly provides, with various outcomes. Given the similarity between HPeVs and HEVs it seems rational to provide IVIGs to neonates/infants with severe HPeV infection as well.[18]

In this case report we describe for the first time an infant with myocarditis and dilated cardiomyopathy due to an HPeV1 infection who was successfully treated with IVIGs.

Patient presentation

A 5-month-old boy was admitted to the PICU with circulatory insufficiency due to suspected myocarditis.

The medical history of the boy was unremarkable until the age of 3 months. He was born at term after an uncomplicated pregnancy. He was growing adequately, but excessive perspiration was visible during feedings. On admission, the patient was pale, markedly dyspneic, and irritable, with a heart rate of 180 beats per minute and a respiratory rate of 40 breaths per minute. His temperature was normal. The patient's oxygen saturation was 94% and blood pressure was 88/58 mm Hg. Examination of the heart revealed soft heart sounds without audible extra heart sounds, no mitral regurgitance murmur and an enlarged heart with percussion. The lungs and abdomen revealed no abnormalities. The chest radiograph revealed an enlarged heart. The electrocardiogram showed sinus tachycardia, normal axis, and abnormal repolarisation with no signs of ischemia.

The first laboratory evaluation revealed elevated cardiac enzymes without other deviations or signs of infection (Table 1).

An echocardiogram was performed and showed a severely dilated left ventricle with a shortening fraction of 3% and a left ventricle end- diastolic diameter (LVED) of 202% (Fig. 1). No structural abnormalities possibly leading to left ventricular failure were detected, suggesting a viral cause of the dilated cardiomyopathy.

Supportive treatment with milrinone, dobutamine and diuretics was started. A 3-day course of 2 g/kg IVIGs was started because a viral cause of myocardiopathy was suspected and no other treatment options were available. HPeV was detected in blood and feces by 5'untranslated region (UTR) polymerase chain reaction (PCR),[19] which was revealed to be an HPeV1 by molecular typing.[20,21] No other viruses were detected at the time of diagnosis except for a very low cytomegalovirus (CMV) viral load in blood (<1000 copies/mL), which was considered clinically irrelevant. Positive Epstein-Barr virus and CMV IgG antibodies were interpreted as the result of transmission of maternal IgG antibodies. Serological tests for Coxsackievirus B, herpes zoster virus, herpes simplex virus, mycoplasma and mumps were negative. A cardiac biopsy was not performed.

Table 1. Patient's laboratory results on admittance.

Marker	Value
Hemoglobin, mmol/L	6.3
Leukocytes, 10^9/L	11.3
Neutrophils	7.72
Lymphocytes	3.31
Thrombocytes, 10^9/L	415
C-reactive protein, mg/L	1
Sodium, mmol/L	143
Potassium, mmol/L	5.1
Calcium, mmol/L	2.31
Magnesium, mmol/L	0.98
Serum urea nitrogen, mmol/L	4.6
Creatinine, µmol/L	23
ALAT, U/L	16
ASAT, U/L	40
CPK, U/L	64
CKMB, µg/L	6.7
Lactate, mmol/L	3.2
Troponine T, µg/L	0.178
NT-Pro-BNP, ng/L	67106

ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase.
CPK, creatine phosphokinase; CKMB, Creatine kinase MB isoenzyme; NT-pro-BNP, N-terminal pro b-type natriuretic peptide.

Metabolic diseases were evaluated and excluded; genetic diseases causing cardiac disease were considered not likely by a specialist in genetic diseases.

In the following days digoxine and an angiotensine-converting enzyme inhibitor were added to support cardiac functions. One week after the start of IVIG treatment, the patient's clinical condition improved.

Cardiac functions improved with a shortening fraction of 10.5%. Although LVED remained unchanged, a significant decrease in N-terminal pro b-type natriuretic peptide (NT-pro-BNP) from 67 000 to 5000 ng/L implied reduced left ventricular filling pressures. In the following weeks the boy continued to recover and cardiac function improved steadily. After 1 month HPeV could not be detected by PCR in the blood, whereas HPeV shedding in faeces occurred until 2 months after admission.

In addition, HPeV1-specific antibody titers were determined in blood, showing a negative titer (<8) on admittance, an increase in antibody titer to 512 directly after treatment with IVIGs and a residual antibody titer of 32 after 1 month. Earlier results from our laboratory revealed a high titer of HPeV1-specific antibody titers in IVIGs.[22]

Cardiac medication was gradually reduced, and after 6 weeks the boy was discharged. Cardiac function was normalized with a shortening fraction of 32% and an LVED of 115% on echocardiographic follow-up 6 months later.

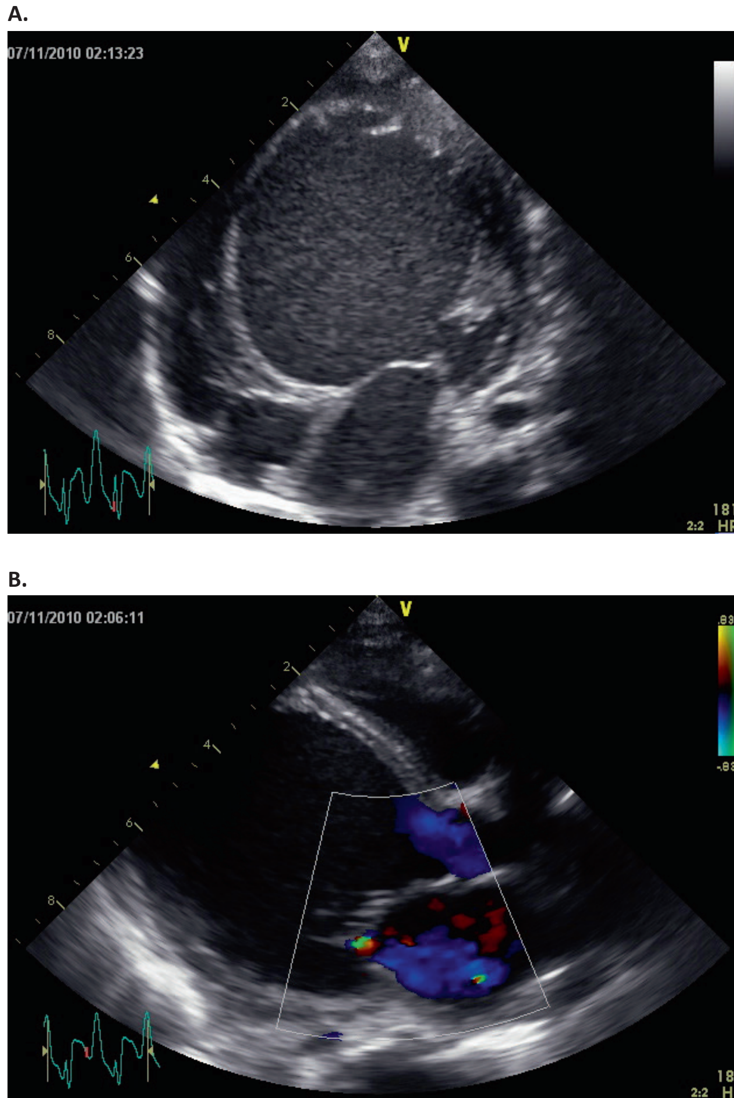

Figure 1. Echocardiography showing a profound dilatation of the left ventricle **(A, B)** and mitral regurgitation **(B)**.

Discussion

In this case report we describe for the first time an infant diagnosed with severe myocarditis and dilated cardiomyopathy associated with HPeV1 who was treated successfully with IVIGs. To our knowledge, myocarditis due to HPeV1 has been described only twice.
Maller *et al.*[9] described a child aged 14 months old who died of myocarditis. Echovirus 22 (later reclassified as HPeV1) was cultured from the myocardium and pericardial fluid. The child was thought to have an immunological deficiency because of recurrent infections in the past and the unusual course of this echovirus 22 infection. Antibody production against echovirus 22 was not measured in this patient.
Russell and Bell[10] described a 6-week-old boy with a myocarditis (diagnosis based on clinical examination and electrocardiogram) treated with digoxin, steroids and antibiotics. The boy fully recovered within 2 weeks. Blood cultures remained negative. A faeces sample taken 13 days after onset of illness showed echovirus 22 (HPeV1). Low titers of antibodies against Coxsackievirus group B (1-6) as well as echovirus 22 were detected on day 11. A subsequent increase in antibody titers against echovirus 22 to >512 suggested recent infection with this particular virus.

There are no uniform diagnostic criteria for myocarditis in the absence of an endomyocardial biopsy. In a recent seminar (described by Sagar *et al.*[23]) a 3-tiered classification is proposed. In the absence of another cause, an otherwise unexplained increase in troponin concentrations, electrocardiographic changes suggestive of acute myocardial injury, or abnormal function on echocardiogram or cardiac MRI in combination with a recent trigger for myocarditis (eg, recent viral illness), the possible diagnosis of subclinical myocarditis can be made. Our patient had a (viral) respiratory infection a week earlier. If a patient meets the above mentioned criteria and in addition has acute heart failure or chest pain or (pre) syncope or myopericarditis, the diagnosis is probable acute myocarditis. If myocarditis is confirmed by histological studies, the diagnosis is definite myocarditis.
Our patient fulfills the criteria for probable myocarditis (the highest diagnostic category without cardiac biopsy). In addition, parechovirus was isolated from the blood and feces, suggesting disseminated viral disease.

HEVs and more specific Coxsackieviruses are well known to cause myocarditis and/or cardiomyopathy in children. However, in a considerable number of patients no direct cause is found. A retrospective study by Arola *et al.*[24] in 33 children en adolescents with end-stage dilated cardiomyopathy revealed a positive PCR for HEV in myocardial material in only 1 patient, although the authors were able to detect cellular mRNA in the samples for 32 patients. In another retrospective study by Martin *et al.*,[25] a viral cause could be detected in 26 out of 38 myocardial tissue samples of 34 children with acute cardiomyopathy. Adenovirus was most frequently detected in 15 samples followed by HEV in 8 samples.

Only HEVs, adenoviruses, herpes simplex virus types 1 and 2, and CMV were tested in this retrospective study. In addition, in adults with myocarditis and/or dilated cardiomyopathy, parvovirus B19 has been found in a number of patients (reviewed in Dennert *et al.*[26]).
The role of different viruses in the pathogenesis of myocarditis in children remains unclear.

Treatment options for acute viral myocarditis/cardiomyopathy are limited. Especially in children, administration of IVIGs is a commonly used treatment option.[27] A systematic review of the use of IVIGs in viral myocarditis reported only 1 randomized controlled trial in adults and some additional studies of various designs. In many cases, a viral cause of myocarditis could not be directly demonstrated and routine usage of IVIGs for acute myocarditis was therefore not recommended.[28]
Freund *et al.*[29] reported on 7 neonates with HEV myocarditis and reviewed the literature. Only 4 of 35 neonates with HEV myocarditis received IVIGs (3 of them were also treated with pleconaril). Two patients died and only 1 patient recovered completely. The mortality rate was 32% and 58% of the survivors developed severe cardiac sequelae.
In a study by Dennert *et al.*[30] in adults with idiopathic cardiomyopathy for at least 1 year, 2 g/kg IVIGs were given to 15 patients with an endomyocardial, biopsy-proven, high parvovirus B19 load. Parvovirus B19 is highly prevalent in the general population and hence a high antibody titer against Parvovirus B19 in IVIGs was expected. A significant decrease in parvovirus B19 load and improvement in cardiac function was seen 6 months after treatment.
We showed *in vitro* that IVIGs contain high titers of neutralizing antibodies against HPeV1.[22] *In vivo*, our patient's blood samples revealed an important increase in HPeV1 antibody titers after treatment with IVIGs. These results are in accordance with the marked clinical improvement of our patient after treatment with IVIGs.
Our hypothesis is that patients with myocarditis caused by viruses with a high prevalence in the population and hence high antibody titers in IVIGs are likely to benefit from treatment with IVIGs. More research combining virological and clinical data is needed to see whether this hypothesis is true.

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Chapter 10

Specific cell tropism and neutralization of human parechovirus types 1 and 3: implications for pathogenesis and therapy development

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Abstract

Human parechoviruses (HPeVs) are picornaviruses frequently infecting humans. While HPeV1 is associated with mild disease, HPeV3 is the cause of neonatal sepsis and meningitis. To test whether *in vitro* replication kinetics of HPeV1 and 3 could be related to pathogenicity, HPeV1 and 3 strains isolated from patients were cultured on cell lines of gastrointestinal, respiratory and neural origin and replication kinetics were measured by real-time PCR. No relationship was found between clinical symptoms and *in vitro* replication of the HPeV1 strains. In contrast, the HPeV3 strains showed faster replication in neural cells and there was a relationship between higher *in vitro* replication kinetics and neuropathogenicity in the patient. Furthermore, HPeV1 could be neutralized efficiently by its specific antibody and by intravenous immunoglobulins (IVIG), while most HPeV3 strains could not be neutralized. In IVIG, very low neutralizing antibody (nAb) titres against HPeV3 were found. Additionally, very low nAb titres were observed in sera of two HPeV3-infected donors, while high nAb titres against HPeV1 could be detected. Our data suggest that the mild clinical course of HPeV1 infection is primarily influenced by strong nAb responses, while HPeV3 might be difficult to neutralize *in vivo* and therefore the course of infection will mainly be determined by *in vivo* cell tropism.

Introduction

Human parechoviruses (HPeVs) are small, non-enveloped, single-stranded positive-sense RNA viruses within the genus *Parechovirus* in the family *Picornaviridae.* HPeV1 and HPeV2 were originally described as echoviruses 22 and 23 within the genus *Enterovirus*, based on their similar cytopathic effect (CPE) on monkey kidney cells.[1] However, sequence analysis showed that they were genetically distinct from other enteroviruses (EVs) and they were reclassified as HPeV1 and HPeV2 in the genus *Parechovirus*. Nowadays, 16 genotypes are known but only HPeV1 to HPeV6 were shown to grow in cell culture.[2-7] While EVs are found in individuals of all ages, HPeVs mainly infect children under 5 years old and are usually associated with mild gastrointestinal and respiratory symptoms. An exception is HPeV3, which was first isolated in Japan from a 1-year-old patient with transient paralysis.[5] HPeV1, HPeV2 and HPeV4-6 are all mainly associated with milder symptoms; HPeV3 infections cause central nervous system (CNS) infections and neonatal sepsis.[8-12]
The differences in clinical manifestations between HPeV types may be explained by differences in biological characteristics, such as cell tropism. HPeV3 is more difficult to grow in cell culture than HPeV1.[7,9,13] HPeV3 lacks the arginine-glycine-aspartic acid (RGD) motif located in the C terminus of the capsid protein VP1, which has been shown to be essential for HPeV1 receptor binding and entry.[14-16] The differences in *in vitro* growth characteristics [7,13] and the lack of the RGD motif imply use of a different cellular receptor by HPeV3 from that used by HPeV1, and a potentially different cell tropism. In addition, we showed that frequent recombination occurs between the HPeV genotypes except for HPeV3.[17] This may also imply a different cell tropism of HPeV3, as infection of different cell types *in vivo* might reduce the opportunity for recombination to occur. A relationship between clinical manifestations and growth characteristics *in vitro* has previously been suggested for EV71. Virus growth was monitored by measuring viral RNA in cell-culture supernatant, showing a fivefold increase of viral RNA 48 h post-infection (p.i.) of EV71 isolated from a patient with encephalitis on the neural cell line SK-N-SH.[18] A second study showed that the encephalitis EV71 strain exhibited better growth on PBMCs and astrocytes than the EV71 strain from a patient without CNS symptoms.[19]
In addition to differences in clinical symptoms, children infected with HPeV3 are significantly younger (<2 months) than children infected with HPeV1 (>6 months).[8,20] This might suggest a lack of protection by maternal neutralizing antibodies (nAb) in newborns against HPeV3. For HPeV3, the seroprevalence among women of child bearing age in Japan is 68%,[5] which is lower than the seroprevalence in Finland for HPeV1 in adults (99%).[21,22] For EVs, it has been shown that lack of maternal antibodies is a risk factor for severe disease in infants.[23] In neonates with severe EV infection, intravenous immunoglobulins (IVIG) are used as treatment. Currently this is the only available treatment against severe EV infections, although evidence on its efficacy is lacking.[24]

In this study, we investigate *in vitro* replication kinetics of HPeV1 and HPeV3 virus strains by measuring viral RNA in the supernatant of a range of cell lines by real-time PCR, and relate the kinetics to disease severity. In addition, neutralization capacity of available type-specific antibodies and IVIG was tested *in vitro* to study protection against HPeV1 and HPeV3 infection.

Results

Replication kinetics of HPeV1 and HPeV3 patient strains

To investigate a correlation between pathogenicity and *in vitro* infectivity, replication kinetics of HPeV1 and HPeV3 strains isolated from the stools of patients with documented clinical symptoms were studied on cell lines obtained from human tissues that, given the clinical manifestations, the different HPeV strains might infect: respiratory (A549, HEL, RD99), gastrointestinal (HT29 and Caco-2) and neural (SH-SY-5Y) cell lines (Table 1; Fig. 1). Vero and buffalo green monkey kidney (BGM) cell lines were included as a positive control to test input and replication capacity of the clinical isolates (data not shown).

Table 1. HPeV1 and HPeV3 clinical isolates from patients with differing clinical syndromes.

Patient*	Age (months)	Symptoms			
		GI	Resp	SLI	CNS
P1-1	4.7	+	-	-	-
P1-2	9.9	+	-	-	-
P1-3	6.0	-	+	-	-
P1-4	5.6	+	+	-	-
P1-5	4.8	+	+	+	-
P1-6	0.7	-	-	+	+
P3-1	0.9	+	-	-	-
P3-2	10.6	+	+	-	-
P3-3	0.2	-	-	+	-
P3-4	10.8	+	-	-	+
P3-5	1.0	-	-	+	+
P3-6	0.2	+	+	+	+
P3-7	1.7	+	+	+	+

GI, Gastrointestinal symptoms; Resp, respiratory symptoms; SLI, Sepsis-like illness; CNS, central nervous system symptoms.
*Numbers starting P1 and P3 indicate HPeV1- and HPeV3-infected children, respectively.

Six HPeV1 strains and seven HPeV3 strains isolated from 13 stool samples were selected according to the clinical manifestations and severity of disease documented for the patients (Table 1) and were cultured with the same input per cell culture (m.o.i. 0.001). Of the HPeV1-

infected children, patients P1-1 to P1-4 showed mild gastrointestinal and/or respiratory symptoms; P1-5 in addition had sepsis-like illness (SLI) and P1-6 suffered from SLI with CNS involvement.[8,20] In the HPeV3 group, P3-1 and P3-2 had mild disease without SLI or CNS symptoms, P3-3 had SLI without CNS involvement, P3-4 had CNS infection without SLI, and P3-5, P3-6 and P3-7 had SLI and CNS involvement.

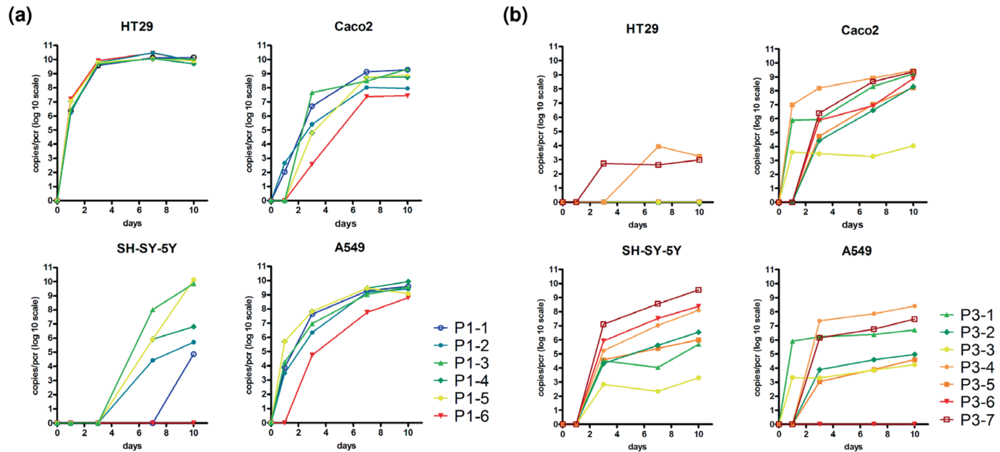

Figure 1. Replication kinetics of HPeV1 and HPeV3 patient strains on HT29, Caco-2, SH-SY-5Y and A549 cell lines. The HPeV strain numbers correspond with the patient numbers in Table I. **(a)** Replication kinetics of HPeV1 strains. P1-1, P1-2, P1-3 and P1-4 were isolates from patients with mild gastrointestinal and respiratory symptoms (green/blue), P1-5 from a patient with SLI (yellow), and P1-6 from a patient with SLI and CNS symptoms (red). **(b)** Growth kinetics of HPeV3 strains. P3-1 and P3-2 were isolated from patients displaying only mild symptoms (green/blue), P3-3 was isolated from a patient with SLI (yellow), P3-4 was isolated from a patient with CNS symptoms and P3-5, P3-6 and P3-7 were isolates from patients with both SLI and CNS symptoms (orange/red). Cells were infected with HPeVs at an m.o.i. of 0.001 and viral RNA was detected in the supernatant with RT-PCR. The $\log_{10}$ virus copies were calculated using a standard curve, and the input virus copies per PCR at time point 0 were subtracted.

All HPeV1 and HPeV3 strains were able to replicate on the positive-control cell lines Vero and BGM (data not shown). P1-1 to P1-6 strains all showed remarkably similar and highly efficient replication kinetics on the HT29 cell line, with high levels of replication reaching $>10^{10}$ copies per PCR at day 10 p.i. (Fig. 1a). While replication on the HT29 cell line was absent for five HPeV3 strains (P3-1, P3-2, P3-3, P3-5, and P3-6), we did observe low-level replication ($<10^4$ copies per PCR) for P3-4 and P3-7, both strains from patients with CNS symptoms (Table 1; Fig. 1b). Remarkably, all HPeV3 strains were able to replicate on the other gastrointestinal cell line, Caco-2, with the same high titres as shown for HPeV1, with the exception of strain P3-3. Replication of both HPeV1 and HPeV3 strains was supported by the A549 and HEL cell lines, with the exceptions of strain P3-6 (A549) and strain P3-5 (HEL). The HPeV3 strains showed slower replication kinetics, reaching lower viral titres than HPeV1 strains on the A549 cell line (Fig. 1a, b), while all HPeV strains showed slow growth

on the HEL cell line (data not shown). HPeV1 strains P1-1 to P1-5, isolated from the stools from patients with no signs of CNS involvement, did show replication on the neural cell line SH-SY-5Y, albeit with slow kinetics, starting replication at day 3 p.i. P1-6, isolated from the stool from a patient with CNS symptoms, was not able to replicate on the SH-SY-5Y cell line. In contrast, rapid replication on SH-SY-5Y cell line was observed for all HPeV3 strains. P3-4, P3-6 and P3-7, isolated from the stools from patients with CNS symptoms, showed up to a threefold higher viral RNA titre at day 10 p.i., compared with P3-1, P3-2 and P3-3 that were isolated from the stools from patients without CNS symptoms; however, P3-5 did not reach these higher titres despite being isolated from a patient with CNS involvement (Table 1; Fig. 1b).

Neutralization capacity

Polyclonal antibodies available against HPeV1 and 3 were tested for neutralization capacity against HPeV1 to HPeV6 laboratory prototype strains by inhibition of CPE in cell culture (Table 2). The anti-HPeV1 (aHPeV1) antibody neutralized its respective genotype without cross-neutralization of other types, as expected (Table 2). Remarkably, our laboratory prototype HPeV3-150237 was not neutralized by the anti-HPeV3 (aHPeV3) antibody directed against the Japanese prototype HPeV3 A308-99 strain (Table 2). Therefore, we compared the neutralization capacity of the HPeV3 antibody against the original HPeV3 strain A308-99 from Japan and the HPeV3 strain P3-2 as well. To confirm neutralization, an immunofluorescence assay (IFA) was used to measure whether there was viral infection present within the cell. Neutralization could be shown against A308-99, albeit at a very low titre of 1:10. The HPeV3 clinical isolate P3-2 could not be neutralized (Fig. 2). With IFA, it could be shown that the aHPeV3 antibody did bind to HPeV3-150237, as well as to HPeV1-Harris (Fig. 3). In contrast, the aHPeV1 antibody bound HPeV1 exclusively.

Table 2. Neutralization of HPeV1 to HPeV6 by polyclonal antibodies aHPeV1 and aHPeV3, measured by CPE at day 7 p.i.

HPeV strain		**Antibody**	
	aHPeV1*	**aHPeV3†**	**None**
HPeV1 Harris‡	-	4+	4+
HPeV2-751312‡	4+	4+	4+
HPeV3-150237‡	3+	3+	3+
HPeV4-251176‡	4+	4+	4+
HPeV5-552322‡	4+	4+	4+
HPeV6-550389§	4+	4+	4+

-, No CPE; 1+, 0-25% of infected cells showing CPE; 2+, 25-50% CPE; 3+, 50-75% CPE; 4+, 75-100% CPE.
*Antibody dilution for HPeV1 was 1 : 10.
†Antibody dilution for HPeV3 was 1 : 2.
‡100 $TCID_{50}$ infection on HT29 (HPeV1, HPeV2, HPeV4, HPeV5) and Vero (HPeV3) cells.
§1000 $TCID_{50}$ infection of HT29.

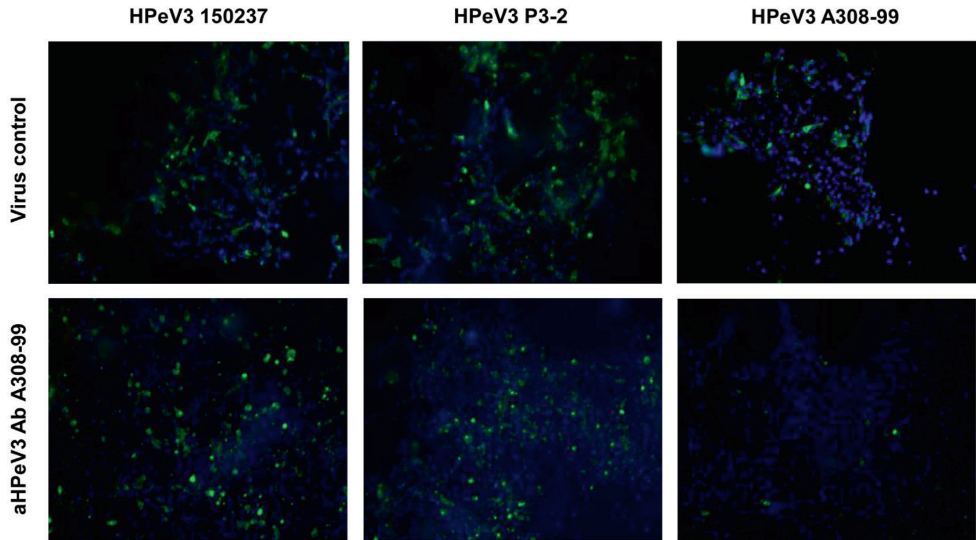

Figure 2. Neutralization ability of aHPeV3 antibody. IFA for detection of HPeV3-150237, A308-99 and P3-2 after HPeV3 A308-99 antibody neutralization. A standard neutralization assay was performed: the HPeV3 strains were pre-incubated with the polyclonal A308-99 antibody and used to infect Vero cells. Cells were stained at day 7 with polyclonal antibody against HPeV3, and secondary FITC-labelled goat IgG antibody (green). The nuclei of the cells are stained with DAPI (blue).

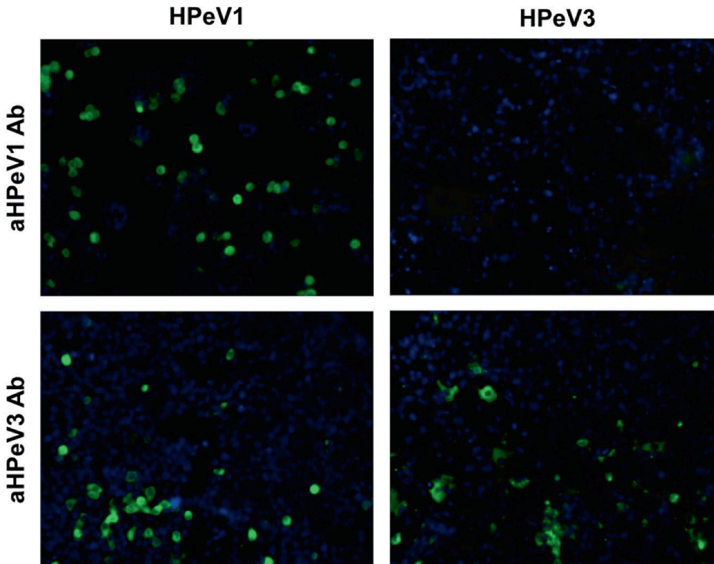

Figure 3. Immunofluorescence showing HPeV1 and HPeV3 antibody binding. IFA for detection of HPeV1 infection in HT29 cells and HPeV3 infection in Vero cells. When a CPE of 2+ (25-50% of cells infected) was reached, cells were stained with polyclonal antibodies against HPeV1 and HPeV3, and secondary FITC-labelled goat IgG antibody (green). The nuclei of the cells are stained with DAPI (blue).

To test the neutralization capacity of IVIG against HPeVs, six different IVIG batches from 2005, 2008, 2009 and 2010 were used for neutralization. High neutralizing titres against HPeV1 were found (>1:1280), while neutralization titres against HPeV3-150237 and HPeV3 A308-99 were very low (<1:20 and <1:40, respectively) (Table 3). In contrast, IVIG contained high nAb titres against the other HPeV genotypes 2, 4, 5 and 6 (>1:320) (data not shown). No substantial differences were shown between the IVIG batches from the different years.

Table 3. IVIG neutralization titres (reciprocal) at day 7 p.i. against the HPeV1 and HPeV3 genotypes.

Genotype	Batch					
	2005	**2008-1**	**2008-2**	**2009**	**2010-1**	**2010-2**
HPeV1 Harris	1280	1280	2560	1280	1280	1280
HPeV3 150237	20	20	10	20	20	10
HPeV3 A308-99	10	20	20	40	40	20

To further investigate neutralization of HPeV3, sera from two different donors, in whom an HPeV3 infection had been detected by PCR in a stool sample from the past year, were tested for neutralization by CPE as well as PCR read-out (Fig. 4). High nAb titres against HPeV1 were found in both donors. Sera of donors 1 and 2 showed nAb titres of 1:256 and 1:4096, respectively, at time point 1, indicating previous infection with HPeV1. After 1 year, the sera of donors 1 and 2 had nAb titres of 1:512 and >1:4096, respectively, indicating that nAbs can still be strong at least 1 year after infection. An 8 $\log_{10}$ difference was measured in viral copies by PCR between non-neutralized and neutralized virus infection (Fig. 4a), and virus growth could only be visualized by IFA starting at the indicated serum antibody dilutions (Fig. 4b). However, for HPeV3 these high nAb titres could not be found after infection. At time point 2, 1 year after infection, incomplete inhibition of virus growth could be shown by an antibody titre of 8, while no growth inhibition could be found by antibody titres of 32 and upwards (Fig. 4b). Virus replication measured by PCR decreased with a maximum of 2 $\log_{10}$ only at the very low nAb titres (<1:16) (Fig. 4a). Therefore, in two adult donors 1 year after HPeV3 infection, partial neutralization can be found only at very low serum antibody titres (<1:16), which is in contrast to the high serum nAb titres against HPeV1 found in these two adult donors without apparent recent HPeV1 infection.

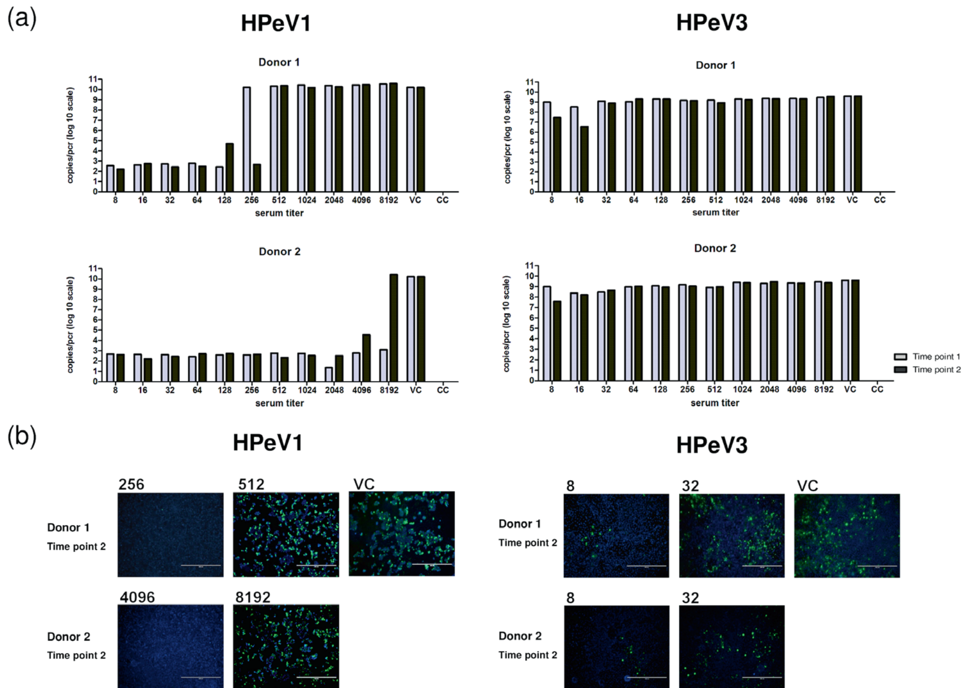

Figure 4. Neutralization of donor serum samples. An end-point neutralization assay of four different serum samples from two different donors who experienced an HPeV3 infection. Time point 1 serum is taken at the time of infection and time point 2 serum 1 year after infection. **(a)** The viral copies per PCR present at day 7. **(b)** Cells were stained at day 7 with polyclonal antibody against HPeV3, and secondary FITC-labelled goat IgG antibody (green). The nuclei of the cells are stained with DAPI (blue). CC, Cell control; VC, virus control.

Discussion

Differences in disease manifestations and severity between EV types can be related to differences in cell tropism and antibody neutralization capability.[18,19,23] In this study, we used PCR to show differences in replication kinetics of HPeV1 and HPeV3 strains from patients with known clinical symptoms. The HPeV3 strains showed better replication kinetics on the neural cell line SH-SY-5Y. Virus strains isolated from three of four patients with CNS involvement showed better replication on the neural cell line SH-SY-5Y, reaching up to threefold higher viral titres, than viruses from patients without CNS involvement. For HPeV1, a correlation between *in vitro* replication dynamics and disease severity could not be found. Of note, these strains have all been isolated from stool samples and therefore do not necessarily represent the key features needed for neural tropism. Interestingly, HPeV3 was able to replicate well on the Caco-2 cell line, while only two strains could replicate (inefficiently) on the HT29 cell line. Caco-2 cells are known for their possibility to differentiate to a more fetal-like phenotype rather than adult ileal enterocytes, resulting in different receptor

expression.[25,26] This phenotype apparently is more suitable for HPeV3 replication than the phenotype of the other gastrointestinal cell line, HT29. Given the lack of the RGD motif of HPeV3, it is likely that HPeV3 uses a different receptor from HPeV1. However, more research is needed to elucidate the mechanisms of differential cell tropism. The cell lines used in our study are continuously growing cell lines obtained from tumours that do not necessarily represent the tissue from which they originated. Receptor expression and post-entry translational mechanisms might differ from their *in vivo* counterparts, which is a limitation for pathogenesis studies. To get better representation of the *in vivo* replication, primary cell systems need to be established.

For picornaviruses, the interaction of the antibody with the RGD motif has shown to be important for virus neutralization.[27] For HPeV1 it has been shown that, next to VP1, VP0 also contains important antigenic sites.[28,29] We showed that our HPeV3 strains could not be neutralized by the polyclonal specific antibody elicited against the Japanese HPeV3 prototype, while the antibody did neutralize the Japanese strain, albeit at a lower titre than reported previously. This could indicate that the antibody has partly lost its potency. Interestingly, the Japanese HPeV3 strain A308-99 is genetically very similar to our strains (97–100 %).[3,17,30] Subsequently, IVIG was tested for HPeV nAb titres. IVIG represents IgGs from >1000 donors and is sometimes given to reduce the disease burden from severe EV infection.[24] IVIG manufactured between 2005 and 2010 by our local blood bank contained high nAb titres against HPeV1, but could hardly neutralize HPeV3 strains, showing that IVIG is not suitable as an antiviral treatment against HPeV3. One could argue that HPeV3 is a newly circulating type and thus there is no high antibody immunity against HPeV3 within the population. However, HPeV3 has been shown to be the second most circulating type since the beginning of the twenty-first century [13,31] and has been present since 1994.[30] Finally we observe very low nAb titres in serum of two HPeV3-infected donors, while high nAb titres against HPeV1 could be detected. A limitation to the study is that neutralization experiments were performed on different cell lines, since optimal HPeV1 and HPeV3 replication is found on different cell lines. From our data we conclude that HPeV3 is difficult to neutralize *in vitro*; whether this is the case for the *in vivo* situation needs to be elucidated further. Based on our observations that HPeV3 is associated with severe disease in young infants, we previously suggested a lack of maternal protection against HPeV3 infection.[8,24,32] Based on the low nAb titres found against HPeV3, despite efficient antibody binding, we now hypothesize that antibody protection against HPeV3 might fail. Therefore, other host and/or viral factors must be involved in susceptibility too, or in protection against infection, and these factors will determine the clinical course of HPeV3 infection in young infants. It could be that the virus structure does not permit antibodies to reach the neutralizing epitope. New mono- and polyclonal antibodies are needed to elucidate the mechanisms of neutralization of HPeV3 and get more insight into potential antiviral drug targets.

In summary, a relationship between replication efficacy on neural cell lines and CNS infection for HPeV3 was found, while this could not be shown for HPeV1. In addition, HPeV3 was

difficult to neutralize with its specific antibody, serum antibodies and IVIG, while efficient neutralization with high titres was found for HPeV1 with aHPeV1 antibody, serum and IVIG. Our study provides new insights into the cell tropism and antibody protection of HPeVs, which are important factors for disease severity. These new insights address the need for antivirals against HPeVs and are essential in the development of treatment strategies and development of these antivirals.

Methods

Cell lines

For virus culture, the following cell lines were used: human colon carcinoma (HT29), human colon adenocarcinoma (Caco-2), human lung carcinoma (A549; kindly provided by the University Medical Center, Leiden), human embryonic lung cells (HEL), rhabdomyosarcoma (RD99; kindly provided by the Dutch National Institute for Public Health and the Environment), African green monkey kidney (Vero), buffalo green monkey kidney (BGM), Rhesus monkey kidney (LLCMK2; kindly provided by the Municipal Health Services, Rotterdam) and human neuroblastoma (SH-SY-5Y; kindly provided by Dr Tauriainen, University of Tampere, Finland). The cells were maintained in Eagle's minimum essential medium (EMEM; Lonza) supplemented with L-glutamic acid (0.2x; Gibco), non-essential amino acids (1x; Gibco), streptomycin (0.1 µg ml^{-1}; Riemer) and ampicillin (0.1 µg ml^{-1}; Sigma) for 1 week before being passaged. For HT29, A549, HEL, RD99, Vero, LLCMK2 and BGM cell lines, the medium was supplemented with 8% heat-inactivated FCS (Sigma) and, for the Caco-2 cell line, with 20% heat-inactivated FCS. The human neuroblastoma cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) and supplemented with heat-inactivated 10% FCS, L-glutamic acid (0.2x), non-essential amino acids (1x), streptomycin (0.1 µg ml^{-1}) and ampicillin (0.1 µg ml^{-1}). Fresh medium containing 2% FCS was added to the cells 3-4 days after passaging.

Virus strains and cultivation

The following HPeV strains were used as laboratory prototypes: HPeV1A Harris, HPeV2-751312, HPeV3-150237, HPeV4-251176, HPeV5-552322 and HPeV6-550389.[8,13,33] In addition, a selection of HPeV1 and HPeV3 strains isolated from patients with well-documented clinical syndromes (Table 1) was made. The Japanese patient strain A308-99 was a kind gift from Dr Shimizu, National Institute of Infectious Diseases, Tokyo, Japan.[5] The viruses were passaged two or three times to obtain sufficient virus stocks. HPeV1, HPeV2, HPeV4 and HPeV5 were cultured in the HT29 cell line, HPeV3 in the Vero cell line and HPeV6 in the RD99 cell line and the virus working stocks were stored in aliquots at -80 °C. The virus concentration was determined by the $TCID_{50}$ and calculated by the method of Reed & Muench.[34]

Antibodies

The anti-HPeV (aHPeV) antibody against HPeV1 (Harris) was obtained from a rabbit antibody pool prepared at the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The aHPeV3 (A308-99) antibody was a kind gift from Dr Shimizu, National Institute of Infectious Diseases, Tokyo, Japan, prepared as pooled guinea pig serum.[5]

Virus replication curves

Monolayers of the different cell lines were cultured in 24-well plates (Cellstar) with 1 ml medium and incubated at 37 °C, 5% CO_2. At day 0, the cell lines were infected with HPeV isolates at an m.o.i. of 0.001 in a volume of 200 µl culture medium for 2 h, after which the non-absorbed virus was removed and replaced with 1 ml maintenance medium (2% EMEM, 2% DMEM) and incubated for 10 days. The low m.o.i. is chosen to elucidate the entire process of the infection cycle of HPeV in cell culture. At days 0, 1, 3, 7 and 10, culture supernatant was removed for RNA extraction and quantitative RT-PCR detection. The supernatant (20 µl) was extracted by automatic extraction using a total nucleic acid isolation kit with the MagnaPure LC instrument (Roche Diagnostics). The RNA was eluted in 50 µl elution buffer and reverse-transcribed as described previously.[13] cDNA (5 µl) was used for real-time PCR using a LightCycler 480 (Roche Diagnostics).[13] The virus copies per PCR were calculated using a standard curve as described previously.[35] Virus replication was normalized to the number of virus copies per PCR (input virus, day 0). At day 10, supernatants were genotyped to confirm the input virus strain by VP1 genotyping as described before.[35] All experiments were performed twice with reproducible results.

IFA

Black, clear-bottomed 96-well plates (Greiner) seeded with Vero or HT29 cells were inoculated with a virus solution in 8% EMEM. When a CPE of 2+ (25–50% of cells infected) was observed, the infected cells were fixed with 4% paraformaldehyde (PFA)/PBS for 15 min. The PFA was removed and the cells were washed consecutively three times with PBS, 25 mM NH_4Cl/PBS and PBS. The cells were placed in 1xPBS/0.1% Triton X-100 for 10 min and washed three times with PBS. To avoid unspecific binding, wells were blocked with 1% BSA/PBS for 30 min. The blocking buffer was removed and the cells were incubated with the primary antibodies, aHPeV1 (rabbit, 1:10000; Jackson ImmunoResearch) or aHPeV3 (guinea pig, 1:100; Jackson ImmunoResearch), for 1 h at 37 °C and then overnight at 4 °C. Plates were washed consecutively three times with PBS, 0.1% Tween/PBS and PBS. Either anti-rabbit or anti- guinea pig secondary goat antibody FITC-labelled IgG (15 µg ml^{-1}; Jackson ImmunoResearch) and 0.5 µg DAPI ml^{-1} (Sigma) were added and incubated for 1 h at 37 °C. Plates were washed three times with PBS. The fluorescence was conserved in 50% glycerol/PBS and examined with a fluorescence microscope (Leica).

Neutralization assay

Antibodies were mixed with the different HPeV1 and HPeV3 virus suspensions containing 100 $TCID_{50}$ per 50 µl. The following antibody dilutions in 2% EMEM were used: aHPeV1 (1:100); and aHPeV3 (1:10). Secondly, six different IVIG batches [*Nanogam*, Sanquin, The Netherlands, from 2005, 2008 (two batches), 2009, 2010 (two batches)][31] were used for end-point neutralization of 100 $TCID_{50}$ per 50 µl HPeV1 and HPeV3. IVIG are batches of pooled IgG extracted from plasma of over 1000 donors. An end-point neutralization was performed with serum from two HPeV3-infected donors at two different time points. The first serum sample was taken during the HPeV3 infection and the second sample 1 year after infection. Mixtures were incubated at 37 °C for 1 h, and were used to inoculate HT29 cells (HPeV1) and Vero cells (HPeV3) on a 96-well plate (200 µl). Virus, cell and antibody controls were included as positive and negative controls. The cells were examined for the appearance of CPE every 24 h for 7 days. At day 7, the medium, containing unbound antibody, was removed and then cells were fixed in the 96-well plate and an IFA was performed. For the patient serum neutralization, the virus copies per PCR were measured by real-time PCR.

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Chapter 11

Differences in maternal antibody protection against human parechovirus types 1, 3 and 4 in young infants

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***** Both authors contributed equally to this manuscript***

In preparation

Abstract

Background

Human parechovirus (HPeV) type 3 can cause severe disease in neonates and infants, while HPeV1 generally infects older children and elicits mild symptoms. In the adult population, seroprevalence of HPeV3 antibodies is low compared to HPeV1 and HPeV4. Our hypothesis is that lack of maternal neutralizing antibodies (nAbs) is a risk factor for severe disease in infants with HPeV infection.

Methods

This is a prospective case-control study of mother-child pairs. Cases were children <1 year with a proven HPeV infection (n=38) and their mothers. Controls were children of similar age suspected of a viral infection with a negative HPeV PCR (n=65) and their mothers. Presence of nAbs against HPeV1, -3 and -4 was evaluated by neutralization assays in mothers and children.

Results

HPeV3 infected children (n=22) were younger (median age 1 month) and had more severe disease than HPeV1 infected children (n=8, median age 4 months) and HPeV4 infected children (n=6, median age 5.5 months) ($p<0.05$); one case was infected with HPeV6 and in one cases HPeV could not be typed. Seroprevalence of HPeV1, HPeV3 and HPeV4 in all mothers (n=102) was 99%, 2% and 76% respectively, and did not differ between cases and controls. Mothers of HPeV infected children <3 months (87% HPeV3) had low or absent titers of specific nAbs against the infecting type. HPeV type-specific nAbs against the infecting type were absent in 3 HPeV1 infected and 8 HPeV3 infected case children, while 44% of 16 control children had aHPeV1 nAbs, 12.5% had aHPeV3 nAbs and 38% had aHPeV4 nAbs.

Conclusions

Our results suggest that specific nAbs are protective against severe HPeV infection in the first months of life, but lack in case of HPeV3. Although aHPeV3 nAbs are virtually absent in the population, HPeV3 is almost exclusively eliciting disease in young infants, suggesting a different as yet uncharacterized mechanism of infection and host response in which antibodies do not play a major role.

Introduction

Human parechoviruses (HPeVs) are associated with a wide array of clinical symptoms, ranging from respiratory and/or gastrointestinal disease to life-threatening meningitis/encephalitis, sepsis-like illness (SLI), myocarditis and infant death syndrome.[1-4] Of the current 16 known HPeV types, HPeV1 and HPeV3 are most frequently detected.[5-7] While HPeV1 mainly causes mild gastrointestinal and respiratory disease, HPeV3 is associated with neonatal meningitis/encephalitis and SLI.[3,8-11]

HPeV4 is the third most frequent found HPeV type in the Netherlands,[6,12] and causes mild respiratory and gastrointestinal symptoms, but data on clinical symptoms are scarce.[13] Recently 2 cases of SLI in HPeV4 infected children under 3 months of age were reported.[14]

Treatment options for severe HPeV infections are very limited (reviewed in Wildenbeest *et al.*[15]). Intravenous immunoglobulin (IVIG) is given as treatment with various clinical outcomes.[1] The rationale for the use of IVIG in HPeV infections is based on similarities between HPeV and human enteroviruses (EVs). These virus groups are structurally closely related, both belonging to the *Picornaviridae* family, eliciting similar clinical symptoms. In EV infections the humoral immune response is considered to play a major role in pathogenesis, as shown in patients with X-linked agammaglobulinemia who are at risk for severe and chronic EV infections. In this group of patients the beneficial effect of IVIG has been acknowledged.[16] For neonates, a lack of maternal antibodies is considered a risk factor for developing severe EV infection.[17,18] Maternal IgG antibodies are passed through the placenta during pregnancy and are considered to protect against infection in neonates and young children. The half-life of maternal IgG is around 20 days, so protective effects are most pronounced till the age of about 3-6 months. Upon treatment with IVIG, EV infected neonates had a significant higher titer of neutralizing antibodies (nAbs) and a shorter duration of viremia if IVIG contained a high antibody titer against the infecting EV type, although no significant differences in clinical outcome were seen.[17] Since there are over 100 human EV types it is difficult to predict nAb titers and therefore effectivity of IVIG.

Data on HPeV seroprevalence show that practically all healthy adults (92-99%) have obtained antibodies against HPeV1,[19,20] whereas in Europe HPeV3 seroprevalence is only 10-13% in the adult population.[20] Since HPeV3 infection is predominantly seen in children <3 months, this may be due to the absence of maternal nAbs against HPeV3, in contrast to children with HPeV1 infections, who should practically all be protected by maternal nAbs.[21] Indeed, maternal nAbs seem to play a role in preventing HPeV1 infection during the first months of life,[19] but data are missing for HPeV3 infections and the lesser pathogenic HPeV4. To assess the role of protective maternal nAbs in HPeV1, -3 and -4 infections, we conducted a prospective case-control study of mother-infant pairs. The aim of this study was to determine whether the absence of specific maternal nAbs is a risk factor in the acquisition of HPeV infection at a young age and whether the presence of maternal nAbs is protective against HPeV *in vitro*. This study will add useful information about the importance of nAbs

in the defense against HPeV infections in young children and subsequently the rationale for (specific) antibody therapy in HPeV and EV infection.

Methods

Study design

This study is a prospective multicenter case-control study of mother-child pairs in the Netherlands. The Medical Ethical Committee of the AMC approved the study protocol. Written informed consent was obtained from the parents.

Our hypothesis was that maternal HPeV type-specific Abs would protect against HPeV infection in infants. A power analysis calculated that to verify this hypothesis in a case-control setting, 36 cases of mother-child pairs (22 HPeV3 infected cases and 14 HPeV1/HPeV4 infected cases) had to be included, with 2 controls per case resulting in a power of 93-95%. Based on published data, we assumed that 90% of the control mothers have nAbs against HPeV1. The hypothesis that maternal antibodies could protect against infection would be supported if less than 32% of the case mothers have nAbs. Since HPeV3 seroprevalence data were unknown at the time of the study design, it was estimated (based on prevalence data of HPeV3) that 50% of the control mothers would have aHPeV3 nAbs. To support our hypothesis, nAbs should be detectable in less than 8% of the case mothers. Mother-child cases consisted of children under the age of 1 year with a proven HPeV infection (defined as positive HPeV PCR in any clinical sample) and their biological mothers. Control mother-child pairs were children of similar age undergoing identical diagnostic tests, but with either a proven EV infection or without either EV or HPeV infection, with their biological mothers. Patients <1 year with clinical suspicion of a viral infection with HPeV and/or EV of whom diagnostic virological tests were obtained, were eligible for inclusion in the study. Patients were included between July 2008 and November 2012 in the following hospitals in the Netherlands: Academic Medical Center (AMC), Amsterdam (n=62), Amstelland Hospital, Amstelveen (n=12), Zuwe Hofpoort Hospital, Woerden (n=20), Meander Medical Center, Amersfoort (n=7), University Medical Center Utrecht (UMCU), Utrecht (n=1) and Free University Medical Center, Amsterdam (n=1).

Within 1 week after sampling for routine diagnostic virological tests, stool and blood samples were collected from mothers of the study participants. Results from routine virological tests on stool, blood, cerebrospinal fluid (CSF) and nasopharyngeal aspirate (NPA) were recorded. Exclusion criteria were prematurity before a gestational age of 34 weeks and maternal use of IVIG during pregnancy.

Demographics, data on presence and duration of clinical symptoms, use of antibiotics, the presence and site of isolation of other microorganisms and diagnosis at discharge were recorded from the patient's files and discharge letters.

Virus detection

Stool, CSF, blood and NPA were tested in respectively 99%, 39%, 17% and 24% of the children (Table 1). HPeV and EV double infections in stool samples were included in the HPeV group (6 children), unless EV was detected as the only pathogen in CSF or blood (1 child).
HPeV and EV real-time reverse transcription (RT-) PCR based on the 5' untranslated region (UTR) was performed on available samples (stool, CSF, blood, NPA) as described previously.[12] A cycle threshold (Ct)-value of 40 or more was considered negative.[22] HPeV positive stool samples were genotyped by sequencing the complete VP1 region.[12]

Table 1. HPeV and EV PCR results and frequency of co-infections in available materials of included children.

Group	Feces pos*	CSF pos*	Blood pos*	NPA/throat swab pos*	Other micro-organism (%)
Cases (HPeV pos)	37/38	9/11	7/7	2/10	15/38 (40%)
HPeV1	*8/8*		*2/2*	*1/4*	*6/8 (75%)*[#]
HPeV3	*22/22*	*9/9*	*4/4*	*½*	*4/22 (18%)*[#]
HPeV4	*6/6*	*0/1*		*0/4*	*5/6 (83%)*[#]
HPeV6	*1/1*				*0/1*
HPeV unknown	*0/1*	*0/1*	*1/1*		*0/1*
Controls (EV pos or HPeV/EV neg)	28/64	8/23	6/10	3/14	20/65 (31%)

*PCR positive for HPeV (in HPeV group) or EV (in control group), displayed as number positive / tested material for HPeV/EV PCR.
[#]$p<0.05$ for HPeV3 versus HPeV1 and HPeV4.

Detection of neutralizing antibodies

Neutralizing antibodies against HPeV1, -3 and -4 in children and mothers were tested using an end point neutralization assay as previously described.[23] If children received IVIG, only blood samples before IVIG treatment were evaluated. For the neutralization of HPeV3, Vero cells were used since HPeV3 was reported to replicate sufficiently in this cell line.[24] All samples were tested with the HPeV3-150237 strain, while part was also tested with the HPeV3-51930 strain. For the neutralization assays of HPeV1 and -4 HT29 cells were used with the HPeV1 Harris strain and HPeV4-K251176 strain respectively. The reported antibody titer was defined as the highest serum dilution able to prevent cytopathic effect (CPE) formation. An antibody titer of 1:8 or more was considered positive. Each maternal sample was tested twice and the mean titer was used for analysis. The highest dilution was used in the event of one dilution difference between titers. The end result was reported as positive if one test yielded a low positive antibody titer (1:8 to 1:16) while the other test had a negative result (n=9). Reproducibility between the two independently performed tests was high, leading in 99.5% of the results in less than 2 dilution difference in antibody titers.

Statistical analysis

Data were analysed using SPSS for windows, version 20. Categorical variables were compared by means of chi-square test or Fisher's exact test if expected numbers were too low. Differences between normally distributed continuous variables were compared using student-t test and one-way ANOVA. For unequally distributed continuous variables the Kruskall-Wallis and Mann-Whitney U test were used. Spearman's rank correlation was used to test correlations. A p-value <0.05 was considered to be significant.

Results

General description of cases and controls

In total 103 mother-child pairs were included; 38 case (HPeV infected) children (including one twin) and their mothers and 65 control children (29 EV infected children and 36 HPeV/EV negative children) and their mothers. The cases were divided into children infected with HPeV3 (n=22, 58 %), HPeV1 (n=8, 21%), and HPeV4 (n=6, 16%); one child was infected with HPeV6 and one child with an undetermined HPeV type. These latter two cases were not included in the analyses.

Clinical characteristics of the case and control children are described in Table 2. There was no significant difference in epidemiological characteristics between cases and controls. SLI occurred more frequently in HPeV infected children than in controls. IVIG treatment was given to 3 patients because of severe disease (one HPeV positive and two HPeV negative children).

Differences in age and disease severity between HPeV types

HPeV3 infected children were significantly younger (median age 1 month) than HPeV1 and HPeV4 infected children (median age 4 months (p=0.001) and 5.5 months (p=0.012), respectively) (Figure 1A). Of the HPeV3 infected children, 82% (18/22) were <3 months of age, and 41% were neonates (<28 days old). HPeV1 and -4 infections did not occur in neonates. Only 13% (1/8) of the HPeV1 infections and 33% (2/6) of the HPeV4 infections occurred in infants <3 months. Severe infection was diagnosed in 50% (11/22) of HPeV3 infected children (all <3 months), compared to 14% of HPeV1 (2/8, one <3 months) and HPeV4 (0/6) infected children (p=0.039). Children with severe disease were significantly younger (median 21,5 days (IQR 10-75 days) than children with mild disease (median 102 days, IQR 41-192 days, p=0.003).

Table 2. Characteristics of cases (HPeV positive) and controls.

Characteristic	Cases	Controls	P-value
Number of children	38	65	
Male/female	1.5:1	1.95:1	0.57
Median age in days (range)	66.5 (5-352)	53 (0-350)	0.81
Underlying disease (%)	7 (18%)	13 (20%)	0.85
Premature (%)	3 (8%)	7 (11%)	0.63
Neonate (%)	10 (26%)	24 (26%)	0.99
Median duration of hospitalisation in days (range)	5 (0-83)*	5 (0-195)#	0.89
Clinical symptoms			
Fever	28 (74%)	52 (80%)	0.46
Sepsis-like illness	13 (34%)	11 (17%)	<0.05
CNS symptoms	24 (63%)	35 (54%)	0.36
Gastrointestinal symptoms	27 (71%)	37 (58%)	0.18
Respiratory symptoms	13 (34%)	14 (22%)	0.16
Skin symptoms	12 (32%)	17 (27%)	0.62
Other micro-organisms found (%)	13 (34%)	20 (31%)	0.72
Use of antibiotics (%)	22 (58%)	41 (63%)	0.60
Median duration of antibiotic treatment in days (range)	3 (1-21)	3 (1-42)	0.58
IVIG treatment	1 (3%)	2 (3%)	0.90
Mean age mother in years (+/-SD)	31.6 (+/-4.9)	31 (+/-5.0)	0.55
Symptoms mother	8 (23%)	13 (21%)	0.83
Feces pos mother/total tested	7/24 (29%)	8/38 (21%)	

CNS, central nervous system; IVIG, intravenous immunoglobulin.
*documented in 32 children, # documented in 60 children.

HPeV1, HPeV3 and HPeV4 neutralizing antibodies in mothers of cases and controls

HPeV1 nAbs were detected in 99% (101/102), HPeV3 in 2% (2/102) and HPeV4 in 76% (78/102) of the mothers, including both the mothers of cases and controls. No significant differences in seroprevalence against HPeV1, -3 and -4 between mothers of cases and controls were found (Figure 2A).

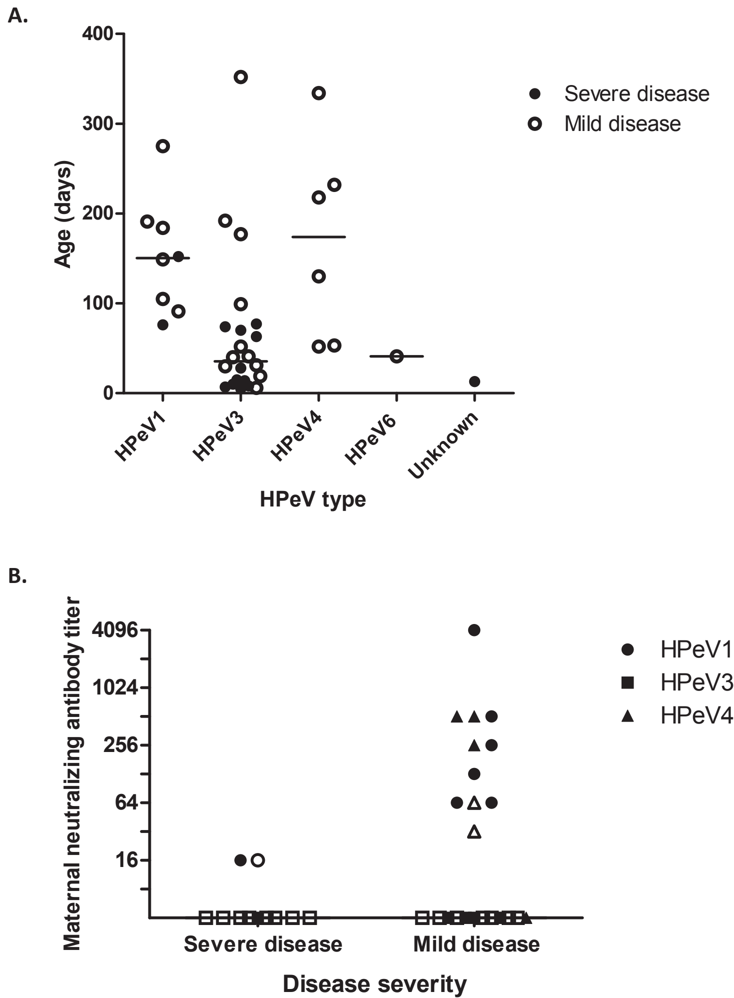
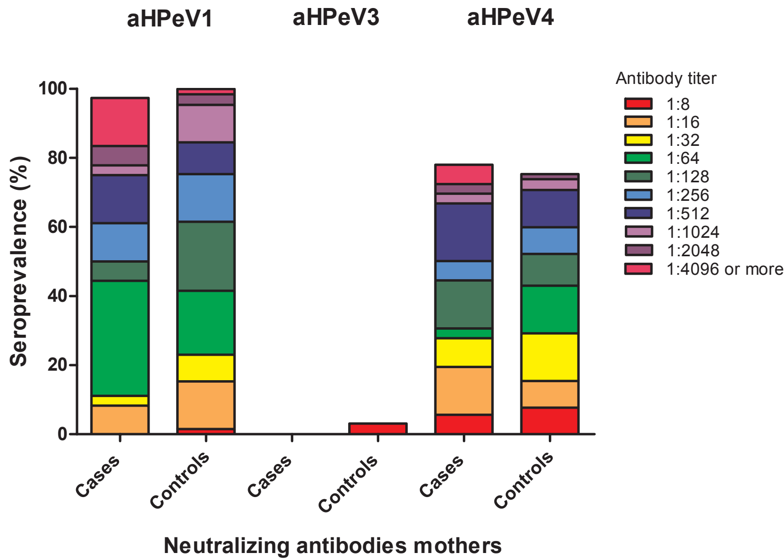


Figure 1. Relation between age, disease severity and maternal neutralizing antibody (nAb) titers in HPeV infected children. **A.** Relation between age and disease severity per HPeV type. **B.** Severity and age of infection (open symbols <3 months of age, closed symbols >3 months of age) in relation to maternal nAb titers against the infecting HPeV type for HPeV1, -3 and -4 infected children.

A.



B.

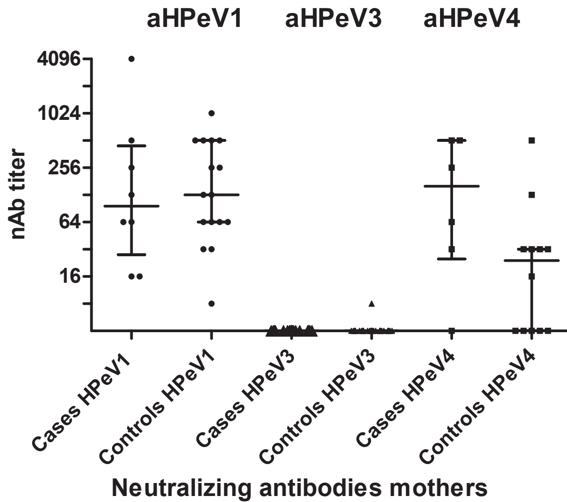


Figure 2. Neutralizing antibody (nAb) titers against HPeV1, -3 and -4 in mothers of cases and controls. **A.** NAb titers against HPeV1, -3 and -4 in the total group of included case and control mothers. **B.** NAb titers against infecting HPeV types in mothers of cases compared to mothers of controls of same age (2 controls per case, median and IQR).

Because of the significant difference in age of children infected with different HPeV types, we compared the nAb titers against the infecting type of the mothers of the cases with age-matched controls, thereby dividing the case group into groups infected with HPeV1, -3 or -4. As shown in Figure 2B, there was neither any significant difference in type-specific nAb titers against the infecting type between mothers of HPeV1 infected cases (median 1:128, IQR 1:32-1:512) and non-HPeV infected controls (median 1:128, IQR 1:64-1:512), nor for mothers of HPeV3 infected cases (median <1:8 (neg)) and controls (median <1:8 (neg)), or for mothers of HPeV4 infected cases (median 1:256, IQR 1:32-1:512) and controls (median 1:32, IQR <1:8 (neg)-1:32). Thus, HPeV1 and -4 seroprevalence in mothers was generally high, while maternal nAbs against HPeV3 were nearly absent in both case and control mothers, therefore protection of maternal nAbs against HPeV infection could not be proven in our case-control setting including children up to 1 year of age. In addition we related age and severity of disease of HPeV infected children to nAb titers in their mothers; low or absent nAb titers against the infecting HPeV type were found in mothers of children with severe disease and in mothers of children <3 months of age (Figure 1B).

The role of maternal neutralizing antibodies in infants younger than 3 months

Since children older than 3 months of age are less likely to be protected by maternal Abs, we further investigated the protective role of maternal nAbs in neonates and infants <3 months of age. We detected significant correlations; for both HPeV1 and HPeV4 the maternal nAb titer correlated with the nAb titer in their children (n=17) ($p<0.05$, Figure 3). Titers in the children were generally lower. Correlations between maternal and infant HPeV3 titers could not be performed because of low HPeV3 seroprevalence.

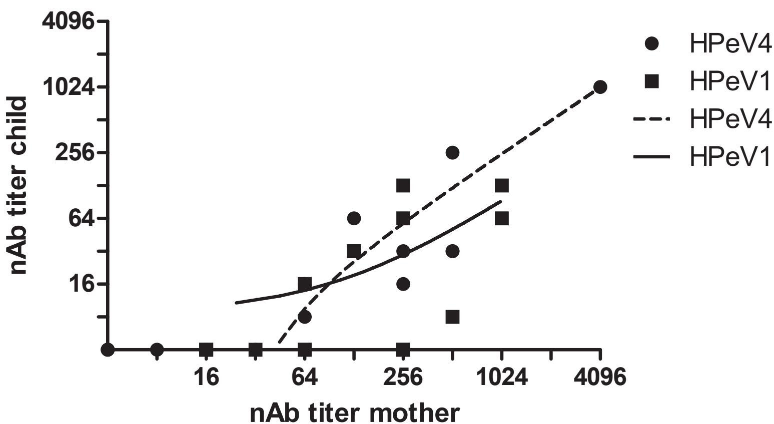


Figure 3. Correlation neutralizing antibody titers of HPeV1 and HPeV4 between mothers and children <3 months of age.

The nAb titers of mothers of cases <3 months of age with HPeV1, -3 or -4 infection (n=21) were compared to nAb titers of mothers of HPeV negative controls <3 months (n=42, Figure 4). Of the 21 cases <3 months, the majority was infected with HPeV3 (n=18), two were infected with HPeV4, and only one child with HPeV1. The mother of the HPeV1 case <3 months had a lower aHPeV1 nAb titer of 1:16 compared to the median titer of 1:128 in mothers of controls <3 months (IQR 1:164-1:512). Mothers of all HPeV3 cases <3 months lacked aHPeV3 nAbs and only 1 of 42 mother of controls <3 months (2.4%) was HPeV3 positive (low titer of 1:8). Two HPeV4 cases <3 months were diagnosed, with aHPeV4 nAb titers of 1:32 and 1:64 in their mothers, as compared to similar nAb titers in mothers of controls <3 months (median: 1:64, IQR 1:8-1:256). Thus, mothers of HPeV infected cases <3 months generally showed absent or low nAb titers against the infecting type.

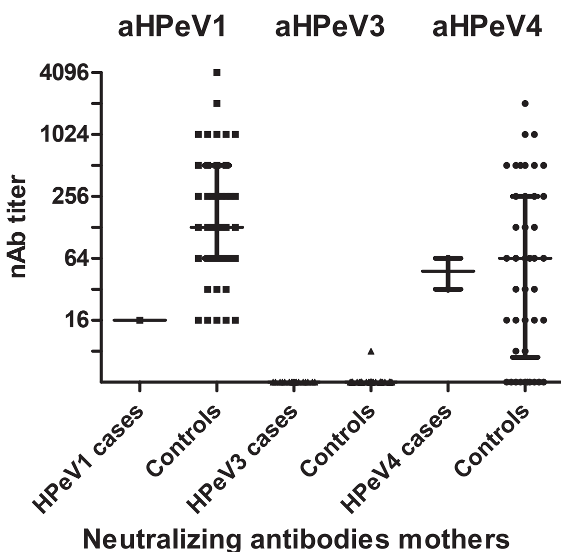


Figure 4. Type specific neutralizing antibody titers in mothers of cases and controls <3 months (median and IQR).

Neutralizing antibodies against HPeV in children

Serum samples from cases – irrespective of their age – were available in sufficient amounts for 8 HPeV3 infected cases, 3 HPeV1 infected cases and 16 control children for testing the presence of HPeV type-specific nAbs. Titers against the infecting HPeV type of the cases were compared with that of HPeV negative controls. In Figure 5 is shown that HPeV1 nAbs were absent in the 3 HPeV1 infected children while in the control group 44% had HPeV1 nAbs, but this was not statistically significant (p=0.26). No differences in nAbs against HPeV3 were seen between cases (0%) and controls (12.5%, p=0.54). In the control group 38% had nAbs against HPeV4, but no blood samples were available from the 6 HPeV4 infected children. Because of the age difference between HPeV1 and -3, we also compared age matched controls with the cases, showing no significant difference in seroprevalence. Although no

significant differences in seroprevalence could be found between cases and controls, none of the HPeV1 and -3 infected children had nAbs against the infecting serotype.

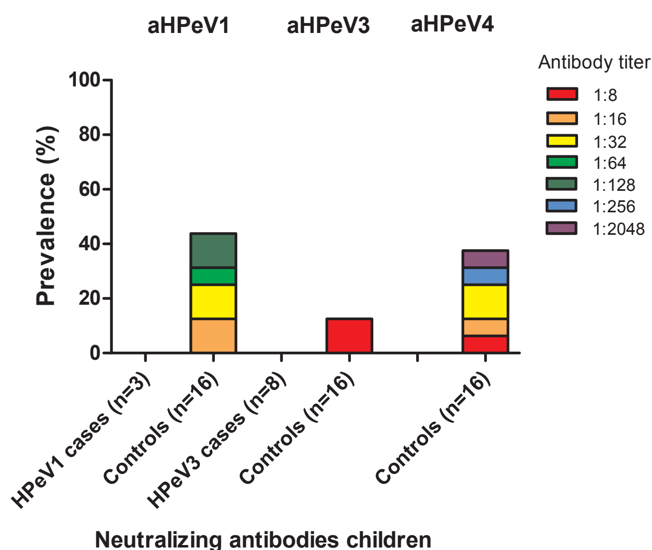


Figure 5. Prevalence of type specific neutralizing antibody titers in case and control children.

Maternal HPeV infection and symptoms

Stool samples of 24 case mothers were available for HPeV PCR testing; 7 (30%) were positive for HPeV (4 HPeV3, 1 HPeV1 and 2 undetermined HPeV types). Only one HPeV3 infected mother, had clinical symptoms (of diarrhea), the rest was asymptomatic. However, four of the HPeV3 infected children of these infected mothers had severe disease as neonates (SLI, meningitis and encephalitis).

Discussion

This is the first case-control study on the protective effect of maternal nAbs against HPeV infections in young children. As only a few HPeV genotypes are commonly circulating in the general population, this virus group should be suitable to study the effect of maternal antibodies in this study design. In contrast, the closely related group of EVs has never been studied in a case-control setting.

Neutralizing antibodies against HPeV1 were found in almost all mothers (99%), while nAbs against HPeV3 were found in only 2% of the mothers. NAbs against HPeV4 were found in 76% of the mothers. Although we could not find significant differences in the presence and titers of nAbs against HPeV1, -3 and -4 between the groups of case and control mothers (because of presence of aHPeV1 nAbs and absence of aHPeV3 nAbs in virtually all mothers),

our results do indicate that the presence of maternal HPeV nAbs protect against (severe) disease in infants. This is illustrated by the fact that HPeV1 infection did not occur at young age. Nearly all infants <3 months of age were infected with HPeV3, with their mothers lacking nAbs. HPeV3 infected children were significantly younger and had more severe disease than HPeV1 and -4 infected children who were in general older than 3 months of age, as reported in previous studies.[4,7,10,25] In addition, in HPeV1 infected infants, clinical symptoms occurred only in the absence of (maternal) nAbs. Therefore our data indicate that young children benefit from maternal nAb protection against both HPeV1 and -4. Cross-neutralization of nAbs between HPeV3 and other HPeV types was not likely based on this and earlier data.[24]

The high seroprevalences of HPeV1 (99%) and -4 (67%) found in mothers is in accordance with recent data in the general population of Dutch and Finnish adults.[20] Seroprevalence of HPeV3 was only 2% in our study, the lowest prevalence reported up to now. Low HPeV3 seroprevalence has been reported in the Finnish (13%) and Dutch (10%) adult population previously.[20] The low seroprevalence of HPeV3 nAbs in our study is also reflected in low HPeV3 nAb titers in different batches of IVIG.[23] IVIG contains IgGs from a pool of more than 1000 donors, and thus is a good representation of circulating nAbs in the general population. Our results further implicate that IVIG is not suitable as treatment in HPeV3 infection; this is in contrast to HPeV1, against which nAb titers in IVIG are high.[23] In contrast, in the Japanese population a higher seroprevalence of 73% in adults was found.[26] Accordingly, in the Japanese population, HPeV3 infection in children is seen at an older age (mean age of 1 year) with predominantly mild gastrointestinal and respiratory symptoms,[5] underscoring our hypothesis that maternal nAbs prevent (severe) disease during the first months of life. Although exact data are missing, the higher seroprevalence of HPeV3 in Japan could be due to longer circulation of HPeV3 in this population. The first HPeV3 strains in Europe have been reported in 1994. It is unclear whether circulation in the population was already occurring at that time,[27] but evolutions studies suggest HPeV3 to circulate for more than 100 years.[28]

The reported differences in HPeV3 seroprevalence could also be explained by differences in the techniques used. For this study, a standard neutralization assay based on CPE formation was used to determine the titer of nAbs. For HPeV1 and -4, complete inhibition of growth by absence of CPE formation could be found in the majority of cases. In general, HPeV3 grows slower in cell culture than HPeV1, and CPE is not easily recognized (unpublished data; own observations). Although slower growth was observed in our virus cultures, none of the serum samples was able to completely inhibit HPeV3 growth *in vitro*. To exclude the possibility of genetic drift as an explanation for lack of neutralization, two different virus strains, both closely related to the currently circulating HPeV3 strains, were used in the neutralization assay. In addition, in an earlier study we showed that the Japanese polyclonal specific antibody against HPeV3 could neutralize the Japanese strain, but not our European strains, suggesting differences between strains in nAb binding. However, the Japanese strain

was very similar to the European strains.[23] The exact origin of this differences remains to be elucidated.

Young children seem to benefit from maternal nAbs against HPeV4, since the median age of infection is even higher than in HPeV1 infected children. However, HPeV4 seroprevalence in mothers is substantially lower than HPeV1 seroprevalence in adults.[19,20] In our study, two children were infected with HPeV4 under the age of 3 months and none of the children had severe disease. However, SLI caused by HPeV4 has been previously described.[14]

Several questions remain unanswered. Unlike EV infection, that occurs at all ages, HPeV infection is restricted almost exclusively to young children.[10,12] For HPeV1 this can be explained by the high circulation rate in young children,[19] and hence a high seroprevalence and protection rate against infection at adult age. For HPeV3, seroprevalence in adults is low, suggesting a lack of protection.[20] However, HPeV3 infections have rarely been reported in adults. The only outbreak of symptomatic HPeV3 disease in adults was reported in Japan, where seroprevalence among adults is higher as compared to our data.[26,29] In this study we showed that adults can be infected with HPeV3, although most infections remain asymptomatic. Thus, it is possible that other factors than the humoral immune responses are of significance and predominate in the host defence against HPeV3 infection. Cellular immunity may play an important role in the protection against HPeV infection, in particular against the HPeV3 serotype. Young infants are known to have an immature T cell response.[28] For example, lower cellular immune responses were seen in EV71 infected patients with brainstem encephalitis and pulmonary edema compared to patients with uncomplicated EV71 infection.[30] Little is known about T cell responses to HPeV infection. Another explanation for the predilection of HPeV3 for young infants could be that young children express specific receptors that enable HPeV3 to display a broad tissue tropism leading to systemic infection including CNS involvement, and that with advancing age this receptor disappears or becomes less expressed, protecting older children and adults from severe or symptomatic HPeV3 infection. Such changes in receptor expression have been shown in mouse models for toll-like receptor (TLR) 8, which is abundantly expressed in neurons and axons of the developing brain but is only marginally expressed in the adult brain.[31] This enhanced expression of TLR8 in the developing brain might play a causal role in the development of white matter injury in neonates with HPeV3/EV encephalitis.[32] The exact nature and extent of cellular immunity, mechanisms of entry and receptor use, however, have not been elucidated for HPeV3 or any of the other HPeVs.

Another question that remains unanswered is why only a low number of infants get symptomatic or severe HPeV3 disease when virtually none of them are protected by maternal nAbs. Most neonates and infants <3 months live relatively protected with low risks of contracting infections, compared to older children. After that age, infection does not cause disease as serious as observed in the very young neonates and infants. This can explain why HPeV3 infection is not as wide-spread as for example HPeV1 which is often causing symptoms when contracted at older age and maternal Abs have disappeared. In

fact, the presence of older siblings in the family is considered a risk factor for acquiring HPeV infection in infants.[33, (Lodder *et al., in preparation*)] Polymorphisms in TLRs as part of the innate immune system can also lead to differences in infectious disease susceptibility between individuals and between populations. Indeed, polymorphisms that lead to reduced TLR3 signaling were significant more seen in patients with EV myocarditis or dilated cardiomyopathy as compared to controls.[34] TLR7 and TLR8 mediated responses are involved in the immune response against HPeV1,[35] but the role of TLRs in HPeV3 infection is unclear. As suggested above, the enhanced expression of TLR8 in the developing brain might play a causal role in the pathogenesis of HPeV3 encephalitis in neonates.[32]
In conclusion, our results suggest that (high titers of) maternal specific nAbs are protective against (severe) disease in young children with HPeV infection. These results support the use of specific antibody therapy in (severely) ill children with HPeV or EV infection. HPeV3 nAbs are virtually absent despite circulation of this virus in Europe for at least 20 years and HPeV3 is almost exclusively eliciting disease in young infants, suggesting a different yet unknown mechanism of infection and host response, warranting further research.

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PART III

Summary and Discussion

Chapter 12

Summary

Enteroviruses (EVs) and human parechoviruses (HPeVs) are both members of the *Picornaviridae* family, a large family of small RNA viruses. The *Enterovirus* genus contains both species that do not infect humans, but animals like pigs, cattle and birds, and species that are capable of causing disease in humans. The human EVs are subdivided in the species human rhinoviruses (HRVs) A-C and the EVs A-D and further in over 100 serotypes, causing a wide array of symptoms from mild respiratory and/or gastrointestinal disease to life-threatening infections like meningoencephalitis and myocarditis. The HPeV species is the only species of the genus *Parechovirus* known to infect humans and currently consists of 16 serotypes. The disease spectrum is similar to that of EVs but significant disease is almost exclusively seen in young children. Currently there is no effective treatment against HPeVs and EVs (including HRVs) available.
Since the introduction of new molecular techniques like PCR, numerous new HPeV and EV serotypes and the HRV-C species were discovered. These technology-driven viral discoveries precede our knowledge of interpretation of test results and of associations between clinical disease and laboratory results. In clinical settings the interpretation of positive results is often part of discussion, since the clinical relevance of a positive PCR result is not always clear.
The aim of this thesis is to describe the prevalence and disease spectrum of EVs and HPeVs and to evaluate the need for treatment together with possible options for the development of effective treatment strategies. In the first part of this thesis some important gaps in our current knowledge of relations between positive human picornavirus laboratory test results and clinical outcomes were investigated. The second part of this thesis focused on treatment against EVs and HPeVs: Is there indeed need for such treatment, since most infections seem to be mild or even asymptomatic? If so, what treatment would be best suitable?

The prevalence of HRV in the first years of life in an unselected birth cohort from the Netherlands and relation with severity of respiratory symptoms is described in **Chapter 2**. HRV was the most prevalent virus in respiratory symptomatic (41%) as well as asymptomatic (26%) children, but was found significantly more frequent in symptomatic children. The rate of (viral) co-infection in symptomatic HRV infected children was high (62%). Overall, respiratory viruses were detected in 86% of symptomatic children with 44% having 2 or more viruses. Children with HRV mono-infection had more severe symptoms, but HRV infections were not associated with occurrence of wheezing. HRV-A and -C were the most commonly detected species in symptomatic children while HRV-B was more often detected in asymptomatic children. There was no association between the different HRV species and occurrence of wheezing or severity of disease. Symptomatic HRV-PCR positive children, in particular wheezing children, had a significantly higher viral load than asymptomatic children, however, a cut-off value for symptomatic disease could not be defined.

In **Chapter 3** the clinical relevance of HPeV1 and -3 detection by PCR in stool samples is described. With the introduction of molecular diagnostic methods in clinical virology laboratories, detection of HPeV is reported more frequently. However, the clinical relevance of these positive findings, especially in stool samples, is under debate. In a retrospective study we showed that HPeV3 was seen in younger children and elicited more severe disease, while HPeV1 was associated with mild symptoms in older children, mainly with underlying disease or in combination with other infectious pathogens. The clinical relevance of detection of HPeV1 in stool samples was therefore not always clear, while a positive HPeV3 PCR in stool samples was associated with clinically relevant disease, regardless of the viral load.

The duration of HPeV shedding in stool samples of symptomatic HPeV infected infants was studied in **Chapter 4**. The duration of shedding varied between 4 and 24 weeks (median 8 weeks) and did not differ between HPeV types. Most children were asymptomatic during the period of shedding. In general, the HPeV viral load decreased gradually over time. At the moment of initial diagnosis, HPeV3 infected children had lower viral loads in stool samples than HPeV1 infected children, while they had more severe disease. This shows that even low viral loads of HPeV3 in stool samples can be associated with severe symptomatic disease and are thus clinically relevant. The HPeV viral load in stool samples could therefore not be used as marker for severity of disease nor for symptomatic disease.

In **Chapter 5** the role of water exposure in the incidence of HPeV and EV infections in infants was studied. By means of a questionnaire the exposure to water of infants in the months prior to a symptomatic HPeV or EV infection was assessed and compared to infants without HPeV or EV infection. We could not identify factors associated with the use of environmental water that increased the risk of acquisition of an HPeV or EV infection. However, siblings in the family were potentially associated with a higher risk of acquiring an HPeV or EV infection.

In **Chapter 6** the need for treatment and possible treatment options against HPeV infections are discussed and compared with EV infections. Although most HPeV (and EV) infections only cause mild symptoms, HPeV is capable of causing severe disease with long-term sequelae in similarity with EV. Up to now there is no effective anti-viral agent against HPeV or EV available. The development of antiviral agents is focused on EVs, especially EV71. Because of crucial structural differences between HPeV and EV, a potential effective drug against EVs, will not automatically be effective against HPeVs. Another possible treatment intervention could be antibody-based therapy since the humoral immune response is considered important in EV infections. Intravenous immunoglobulin (IVIG) has been given haphazardly in life-threatening EV and HPeV infections with various clinical outcomes. This variance in outcome is probably resulting from differences in neutralizing antibody (nAb) titers of specific EV and HPeV serotypes in IVIG products. The development of specific monoclonal antibodies could be a feasible option for short-term treatment of life-threatening HPeV infections.

The anti-enteroviral compound that has been evaluated most extensively in clinical trials is pleconaril. This capsid inhibitor has a broad activity against EVs and HRVs. Pleconaril was never approved by the FDA because of possible side effects and concerns about the development of resistance. However, pleconaril has been used on a compassionate use basis in immunodeficient patients with severe EV infection, but its effectivity has not yet been established. In **Chapter 7** we describe the use of pleconaril in two patients with X-linked agammaglobulinemia and a chronic enteroviral meningoencephalitis (CEMA). The first patient, infected with echovirus 11 (E11), did not recover during the period described and CSF remained positive for EV PCR, while the other patient, infected with echovirus 13 (E13), recovered completely. The difference in clinical outcome of the two patients could be subscribed to the difference in *in vitro* pleconaril susceptibility between the two causative echoviruses. *In vitro* studies can thus be helpful in predicting effectiveness of pleconaril against different EV serotypes.

Remarkably, the patient infected with the pleconaril resistant E11 had never been treated with pleconaril before, indicating a naturally resistant strain. In **chapter 8** the possible mechanisms for the occurrence of this natural resistance were tested and discussed. Analysis of the 3D structure of the capsid revealed that pleconaril anchoring was prevented in the hydrophobic pocket. Comparison with other (pleconaril susceptible) E11 strains revealed 64 amino acid substitutions unique for the resistant E11 strain. Using 3D modelling, three of these 64 amino acid substitutions were identified to be involved in the formation of the hydrophobic pocket. These three mutations were considered possible causes for the resistance against pleconaril. In addition pleconaril resistance was induced in a sensitive E11 strain, resulting in 2 other amino acid substitutions. When introduced into a pleconaril sensitive E11 clone, only one mutation (V119M) led to viable and pleconaril resistant clones. These results underline that natural resistance can differ from *in vitro* induced resistance. This is important to consider when evaluating resistance profiles of new anti-enterovirus medication *in vitro*.

Chapter 9 describes the successful treatment with IVIG of an infant with a dilated cardiomyopathy. HPeV1 was the only identified cause of disease. Treatment with IVIG was given and the infant recovered completely. The IVIG batch that was used to treat the patient contained high titers of specific nAbs against HPeV1. In addition, a significant increase in aHPeV1 nAb titers was detected in the cured IVIG treated patient. This case suggests that IVIG could be effective in severe HPeV disease (like myocarditis), if IVIG contains high nAb titers against the infecting serotype.

In **Chapter 10** specific cell tropism of HPeV1 and HPeV3 was studied *in vitro* to explore the differences in clinical symptoms elicited by HPeV1 and HPeV3 infections, with the latter often causing central nervous system (CNS) infection. HPeV3 strains indeed showed faster replication in neural cell lines as compared to HPeV1 strains and there was a relation

between increased *in vitro* replication kinetics and CNS symptoms in the patients from whom the HPeV3 strains were isolated. For HPeV1 no relation was found between clinical symptoms and *in vitro* replication kinetics. Subsequently, various IVIG batches were tested for type specific nAbs, showing that HPeV1 nAbs titers were high in all batches while only very low nAb titers against HPeV3 were found. In addition, 2 HPeV3 infected donors showed low nAb titers both at the moment of infection and a year after infection. This indicates that antibody protection and thus use of IVIG as treatment in HPeV3 infections might fail.

To assess the role of (maternal) nAbs in protection against HPeV infection in infants a prospective mother-child case-control study has been set up (**Chapter 11)**. As cases, infants with a proven HPeV infection and their mothers were included and compared to controls (infants of similar age with a suspected viral infection, but negative for HPeV) and their mothers. We found no difference in seroprevalence of HPeV1 (99%), HPeV4 (76%) and HPeV3 (2%) between the mothers of cases and mothers of controls. Because maternal nAbs are most important in children <3 months we compared type specific nAbs against the infecting HPeV types of the case mothers with the control mothers. Although nAb titers did not differ significantly between case and control mothers, we found only low or absent nAb titers against the infecting HPeV type in the sera of the mothers of children <3 months with HPeV infection (mainly HPeV3). In available sera of HPeV1 and -3 infected children no specific nAbs against the infecting HPeV type were found. HPeV3 infected children were significantly younger and had significantly more severe disease compared to HPeV1 and -4 infected children. The younger age of infection of HPeV3 infected children compared to HPeV1 and -4 can be explained by the observed difference in maternal nAbs against HPeV3 versus HPeV1 and -4. Thus, maternal nAbs protect against disease in young children with an HPeV1 and -4 infection but maternal humoral protection against HPeV3 is missing. Since HPeV3 infection does not seem to elicit a (sustained) nAb reaction, questions remain about the predilection of symptomatic disease for young children.

In conclusion, HPeV and EV are amongst the most prevalent disease-causing viruses in children and elicit a wide range of disease from mild illness to life-threatening infections. NAbs play an important role in the host defense against HPeVs and EVs, but cannot explain fully the differences in pathogenesis of disease of various HPeV and EV types. Differences in receptor usage, viral virulence factors and host related responses of the cellular and innate immune system are largely unknown, but probably play an important role in host defense as well. Knowledge of both the pathogenesis and host response against EVs and HPeVs is essential to develop effective treatment strategies.

12

Chapter 13

Discussion

The first aim of this thesis was to describe the disease spectrum of human enterovirus (EV) (including human rhinovirus (HRV)) and human parechovirus (HPeV) infections in children, as these picornaviruses are one of the most prevalent pathogens causing disease in children. Knowledge on how to interpret the primarily technology-driven (molecular technology based) viral test results is lacking behind today's technological possibilities with regards to detection of picornavirus infections in children. Therefore thorough insights in bridging laboratory results to clinical disease are needed. The second aim was to evaluate the need for treatment against these infections together with available treatment options and the role of neutralizing antibodies (nAbs) as possible effective treatment interventions. In this chapter the results of this thesis are put into perspective of the current knowledge.

Rhinovirus C seems not to be associated with wheezing or more severe disease in the general population

In Chapter 2 we showed that HRVs are the most prevalent viruses in young children with respiratory symptoms in an unselected birth cohort from the Netherlands. Although more severe infection was seen in the subgroup of HRV infected children without other viral co-infection, we did not find an association between wheezing or more severe disease and HRV-C infection. This is in contrast with an abundant number of recent studies, which showed that HRV-C is associated with more severe disease and asthma exacerbations. The majority of these studies were conducted in hospitalized patients suggesting that the disease spectrum differs between hospitalized patients (with severe disease) and the general population (mainly asymptomatic and mild illness). Indeed, other studies in birth cohorts and non-hospitalized symptomatic children did not show any difference in HRV-C related disease severity. Differences in host related factors and subsequently susceptibility for severe disease may contribute to this. Another possibility is that specific HRV-C types are more pathogenic than others and are overrepresented in hospitalized patients. Lee *et al.*[1] found that the virulence of the HRV-B species in general was lower than the virulence of the HRV-A and -C species and that virulence varied between types. In agreement with our study no difference in wheezing or more severe disease between the HRV-C and HRV-A species was found in the birth cohort they studied.
The role of various HRV(-C) types and HRV(-C) induced host responses needs to be studied in order to clarify if variance in virulence of HRV(-C) types or host related factors or both play a role in the differences found between studies in hospitalized patient and population based cohorts. Therefore studies comparing HRV types between hospitalized patients, patients with mild disease and asymptomatic controls in the same period and region are needed.

Clinical relevance of HPeV infection

The clinical relevance of HPeV became evident since the discovery of HPeV3 and the subsequent association with sepsis-like illness (SLI) and meningoencephalitis in young infants. Together with EV, HPeV3 is the most frequent cause of aseptic meningitis in young children. The mere detection of these viruses by PCR in stool samples is insufficient for the determination of viral induced disease as these viruses are also detected in asymptomatic children and long-term (asymptomatic) shedding after symptomatic infection is frequently observed in children (chapter 4). We again confirmed our earlier findings on differences in disease association between HPeV1 and HPeV3 by comparing a larger group of HPeV infected children diagnosed by PCR in stool samples (chapter 3), showing that HPeV3 infected children were significantly younger and had more severe disease than HPeV1 infected children. Interestingly, low viral loads (high Ct-values) can be found in stool samples of HPeV3 infected infants with severe disease, suggesting that a positive HPeV3 is always clinically relevant. This is in sharp contrast to HPeV1 infection, which is mild or symptomless in most children. The clinical relevance of a positive HPeV1 PCR is part of an ongoing discussion. However, we showed that especially children with an underlying disease are indeed at risk to develop symptomatic HPeV1 disease (chapter 3). We also showed that HPeV1 is occasionally capable of causing severe disease (chapter 9), in agreement with previous reports. In addition, HPeV4, which up to now was associated with mild disease,[2] might as well be able to occasionally cause more severe disease.[3] This underlines the importance of HPeVs as clinical relevant viruses that should be incorporated in diagnostics on viral infection in children.

The long-term duration of shedding after symptomatic infection for several weeks to months is one of the reasons for the difficulties in interpretation of positive test results (in daily clinical practice). The gastrointestinal tract is the main site of replication for EVs as well as HPeVs, with the exception of HRVs, for which the respiratory tract is the main site of replication. It is possible that the detection of viral RNA by PCR in the gastrointestinal and respiratory tract is therefore merely representative of viral presence and does not always implicate invasive and therefore symptomatic disease. In addition, PCR does not discriminate between viable and non-viable viral presence. If the virus is found in otherwise sterile samples, for instance blood, CSF, myocard (in the case of myocarditis), this is considered a sign of invasive disease. Another difficulty in the interpretation of a positive stool sample is that stool samples can be incongruent, causing differences in viral load between different samples. However, in most children of our study viral load decreased slowly over time (chapter 4). The reason for long-term EV and HPeV shedding in feces in young children is not determined yet, but is possibly due to the immature immune system at young age. This long duration of shedding allows the virus to spread very efficiently among the population as young children often visit day care and have close contact with other children.

HPeV3; a disease of neonates?

Only very young children become severely ill from HPeV3 infection (chapter 3) and HPeV3 infection is hardly reported in older children and adults. HPeV1 infection is also mainly found in children below the age of 5 years. For HPeV1 this could be explained by the high seroprevalence in older children and adults.[4] However, since HPeV3 seroprevalence is very low in children as well as in adults,[4] other yet unidentified host or viral factors contribute to the development of HPeV3 disease in young infants. We found that mothers can be infected with HPeV3 (chapter 11), although they reported little or no symptoms in contrast to their children who experienced severe disease, suggesting a difference in disease susceptibility for HPeV3 between young children and adults. Another remarkable observation is that HPeV3 seems to behave different in other populations, such as reported for the Japanese population. Japanese seroprevalence is reported to be much higher than in the European population.[5] Indeed, Japanese children are infected with HPeV3 at an older age (mean 12 months) and symptoms are usually mild,[6] suggesting that in the Japanese population maternal nAbs protect against HPeV3 disease in the first months of life. In addition, an outbreak of epidemic myalgia associated with HPeV3 was reported recently in Japanese adults,[7,8] showing that Japanese adults are susceptible for HPeV3 infection.
Although differences in circulating strains of HPeV3 can be responsible for variations in disease in different populations and patients, this is not very likely, since amino-acid similarity is very high (>99%) between contemporary circulating strains in Europe and the original Japanese strain.[9,10] Host-related factors are probably more of influence in causing HPeV3 disease. Until now, little is known about the receptor usage of HPeV3. It could be that HPeV3 uses a specific receptor to disseminate throughout the body which is present in young infants but is down-regulated in older children and adults, explaining the absence of symptomatic disease at older age. The reason that the Japanese population seems to be more susceptible for symptomatic infection can be caused by differences in genetic host factors between populations. For EV71 it is shown that the class 1 HLA-A33 genotype is associated with an increased host susceptibility to EV71. This HLA type is found more frequently in the Asian populations than in Caucasian populations, providing a possible explanation for the high burden of EV71 related disease in Asia.[11] Knowledge of the pathogenesis of HPeV3 infection and the (differences in) host responses is needed to prove if these hypotheses are true and is essential for the development of effective treatment options.

Need for treatment

Although most EV and HPeV infections are mild and self-limiting, these viruses are able to cause life-threatening infections as is reviewed in chapter 6. Life-threatening infections can be either due to specific viral serotypes or specific host factors. Examples are poliovirus

and EV71 infections which can cause considerable neurologic morbidity and mortality. Although an effective vaccine against poliovirus is available since the 1950s, the virus is still not eradicated due to insufficient vaccine coverage and the emergence of vaccine-associated poliomyelitis. Anti-polioviral agents are therefore considered essential to obtain a polio-free world. These agents are needed in addition to vaccination strategies to treat new cases of acute poliomyelitis and eradicate persistent shedding in immunocompromised persons.[12,13] In Asia the recent outbreaks of EV71 with high morbidity and mortality has led to an enormous scientific activity in search for vaccines and effective treatment. This has not been without success, since recently a promising vaccine against EV71 was developed in China.[14] However, additional antiviral treatment against EV71 will probably still be needed in case of vaccine failure and for specific patient groups in whom vaccination is not indicated or effective (for example neonates and patients with primary immunodeficiencies), given the high morbidity and mortality rate due to brain stem encephalitis and acute flaccid paralysis.[13,15] In the Western world research targeting antiviral treatment against EV and HPeV is lacking behind since the importance of EV and HPeV infections in these countries is not recognized. EVs and HPeVs only cause severe disease in a small subset of the population, making it less attractive for pharmaceutical companies to put a lot of money and effort in the development of antiviral drugs against these viruses. Most EV and HPeV infections are indeed benign and transient in young infants. However, this group is at risk for severe infections with permanent sequelae like meningoencephalitis, myocarditis, hepatitis and even infant death.

Patients with primary immunodeficiencies are another patient category vulnerable for severe EV infections. In this thesis we present several cases of severe EV/HPeV infection, among others two patients with the severe condition of chronic enterovirus meningitis in agammaglobulinemia (CEMA). CEMA can cause significant morbidity and mortality in patients with agammaglobulinemia. Since the introduction of regular prophylaxis with intravenous immunoglobulins (IVIG), the risk of acquiring a chronic EV infection has lowered, but severe disease still occurs, particularly if IVIG does not contains sufficient protective nAb titers against the infecting EV type, as illustrated by our patients (chapter 7). Although this is a rare complication, the burden of disease is high in individual patients.

The HRVs are another group of potential candidates for antiviral treatment. HRVs primarily cause the harmless ‘common cold’. However, the disease burden is high due to the high frequency of infection in the general population and the subsequent economical consequences (e.g. sick leave, visits to health care providers, and prescription of drugs). In addition, HRVs (and especially HRV-C) are recently recognized to also cause more severe disease like bronchiolitis, asthma exacerbations and lower airway infections. Similar to influenza, high infection rate and associations with severe disease makes the development of an anti-rhinoviral agent worth the effort for pharmaceutical companies.

The lack of attention from biotech companies is also reflected by the low numbers of clinical trials with regard to anti-enteroviral drugs; six studies were conducted on anti-rhinoviral

drugs, one on anti-poliovirus drugs and two on anti-enteroviral drugs against severe EV infections.[16] There is currently no antiviral medication against HPeV under development.

Until now, none of the above mentioned clinical trials resulted in a successful registration of an anti-enteroviral drug. Of the many potential candidates, the capsid inhibitor pleconaril has been the most promising, although it was never licensed. In chapter 7 we described treatment with pleconaril as a last resort in 2 patients with CEMA and supported the clinical outcome with in *vitro* data on the susceptibility for pleconaril of the infecting EV strains. We showed that effectivity of pleconaril *in vivo* was consistent with *in vitro* susceptibility of the patient's EV strain. Our results show that pleconaril is effective in life-threatening or chronic EV infections, but depends on the infecting EV type since not all EV types are susceptible for pleconaril. The earlier reported variances in outcome are probably the result of this difference in susceptibility between EV types since the infecting serotype was often not known or EV infection was not even proven. *In vitro* evaluation of susceptibility of different EV types can help to define those EV types against which pleconaril is effective.

The occurrence of resistance remains a considerable problem in both pleconaril treatment and drug development. We showed that pleconaril resistance can occur naturally and can be induced in susceptible strains *in vitro* (chapter 7 and 8). Ideally, the EV strain of the patient should therefore be tested for pleconaril susceptibility *in vitro* to predict the efficacy of treatment. The diversity of the *Enterovirus* genus together with the high mutation rate in most EV types make EVs extremely difficult to target effectively with one drug. In similarity with HIV, where combination therapy has been proven to be more effective in both adults and children,[17,18] we and others suggest that a combination of at least two drugs, targeting different stages in the viral life cycle is eventually the best option for an effective anti-enteroviral treatment, minimalizing the risk of selecting resistant strains.[19]

So far, pleconaril is the only anti-enteroviral drugs with proven efficacy and relative few side effects. In the absence of other treatment options (if IVIG treatment failed), withholding the use of pleconaril for patients with severe or chronic EV infections seems not ethical. The problem is that pleconaril is not available anymore, not even on compassionate use basis. Recently a clinical trial on the efficacy of pleconaril has been conducted in neonates with severe EV infections, but results are pending.[20] Meanwhile registration of pleconaril on compassionate use basis could help to make this drug available for selected patients with severe or chronic EV infection.

Although pleconaril seems the most promising candidate to use in severe EV and HRV infections, it is not effective against today's most life-threatening EVs such as EV71 and poliovirus. Many drugs with a variability of potential targets are currently under investigation because of their presumed activity against EV71 but none of these drugs are evaluated in clinical trials.[20] For the treatment of poliovirus, the capsid inhibitor V-073 seems the most potential candidate *in vitro*.[12,16] In addition, the HPeVs, including HPeV3, are not susceptible for pleconaril.[21] No antiviral drugs against HPeV are currently under development. However, since HPeV3 is causing severe disease in neonates and young infants, and little effect of

IVIG is expected because of the lack of specific nAbs, effective treatment for this particular patient group is urgently needed. Therefore HPeVs should be included in the ongoing research for anti-enteroviral drugs.
A relative new approach in search for an effective treatment is to target host cell factors that are essential for viral replication. This strategy might have two major advantages; broad-spectrum activity and a reduced likelihood of the development of resistance. Promising candidates are inhibitors of the host cell factors Hsp90 and phosphatidylinositol 4-kinase III (PI4KIII). These inhibitors indeed show a broad spectrum of anti-enteroviral and anti-rhinoviral activity.[22,23] But a major drawback is the occurrence of resistance against PI4KIII inhibitors,[24] making these agents unsuitable for use as monotherapy.

Antibodies as treatment option

We used HPeV infection in young children as a model to prove or reject the hypothesis that (maternal) nAbs are of importance in EV and HPeV infections (chapter 11). In a case-control study we showed that there were no protective HPeV3 nAbs in mothers of neither cases nor controls. In contrast, HPeV1 nAbs were seen in 99% of the mothers of both cases and controls. Although no differences in seroprevalence were seen between case and control mothers, we found that HPeV3 infected children were mainly under the age of 3 months, while children with HPeV1 infection were significantly older. These results suggest that (maternal) HPeV1 nAbs are important in protection against (severe) disease (because HPeV1 infection is virtually absent in children <3 months) and therefore could serve as a candidate for treatment. Illustratively, treatment with IVIG in an infant with severe HPeV1 associated dilated cardiomyopathy led to a substantial rise in nAb titers and clinical improvement (chapter 9). The used batch of IVIG contained high titers of aHPeV1 nAbs, in line with the observed improvement. IVIG contains immunoglobulins of more than thousand donors and is a good reflexion of the circulating EV and HPeV types in a specific population. But due to variations in circulating EVs and HPeVs between populations and over years, nAb titers vary per batch, hampering an accurate prediction of effect of treatment with IVIG. This could explain the differences in outcomes of critically ill EV infected patients after treatment with IVIG. For example, Chinese adult donors showed to have high titers of EV71 specific antibodies, providing a rationale for treatment with IVIG in case of severe EV71 infections. Retrospective studies indeed demonstrated a beneficial effect of IVIG when given early in the course of EV71 infections and several countries incorporated the use of IVIG in their treatment guidelines.[25] IVIG treatment of children with severe HPeV3 infections is not likely to be effective, since antibody titers are very low in the general population in Europe and thus in IVIG products.[4] A more targeted approach would therefore be the development of monoclonal antibodies.

A remarkable outcome was that virtually none of the mothers had nAbs against HPeV3, while this virus is circulating for at least 20 years,[9] suggesting a different mechanism of infection and protection. Even after a proven HPeV3 infection, no or only partial inhibition of HPeV3 was found *in vitro* in 2 mother-child pairs (own observations), suggesting that other immune responses are of importance in the defense against HPeV3 infection and high nAb titers are not needed to clear the virus. The absence of production of nAbs after HPeV3 infection in mothers can also be due to the fact that the gastrointestinal tract is only colonized with HPeV3 and does not elicit infection by becoming invasive (because specific receptors are missing) and therefore no production of nAbs is needed. However, in children a sustained or temporary rise in aHPeV3 nAbs was seen in some cases,[4] suggesting that nAbs might play a role as well. More research is needed to elucidate the role of nAbs in the host response against HPeV3 infection.

The innate immune system; mistakenly overlooked?

Toll-like receptors (TLRs) are involved in the immune response against EVs, HRVs and HPeVs. TLR8 is thought to play a role in the pathogenesis of white matter injury in HPeV3 and EV central nervous system (CNS) infection in young children. Experiments in mice showed that TLR8 is also localized in neurons and axons of the developing brain and is responsible for the regulation of axonal growth and development only in the perinatal period.[26] Activation of TLR8 leads to release of reactive oxygen, nitrogen species and pro-inflammatory cytokines which are toxic for neural cells.[27] This could explain why HPeV3 and EV related encephalitis is mainly seen in young infants. TLR8 is also involved in immune-responses in patients with Coxsackie virus B (CV-B) induced dilated cardiomyopathy; increased TLR8 levels were related to adverse clinical outcome.[28]

Polymorphisms in TLRs are frequently seen and are considered of importance in the individual susceptibility for infection caused by microorganisms. For example, genetic variations in TLR3 change the host susceptibility for CV-B3 myocarditis.[29] In addition, polymorphisms of promotor regions of cytokines can influence the cytokine release and subsequently the severity of symptoms. Polymorphisms in the promotor region of IL-6 and IL-10 seem of importance in symptom severity of HRV infections [30,31] and probably influence the susceptibility for development of asthma.[32] Studies in China showed that polymorphisms in IL-6, IL-10, IL-17 and IFN-γ play a role in the development of encephalitis in EV71 infected patients.[33-35] These polymorphisms in TLRs and cytokines could explain differences in susceptibility and disease spectrum between individuals and even whole populations. Future research focusing on host responses against EV and HPeV infections is needed to clarify the role of polymorphisms in pathogenesis of disease between different populations.

Conclusions

HPeVs and EVs are amongst the most prevalent disease-causing viruses in children and elicit a wide range of disease from mild illness to life-threatening infections. Treatment is necessary and urgently needed in severe EV and HPeV infections. NAbs play an important role in the host defense against HPeV and EV viruses, but cannot fully explain the differences in pathogenesis of disease of various serotypes of HPeV and EV. Differences in receptor usage, viral virulence factors and host related responses of the cellular and innate immune system are largely unknown but probably play an important role in host defense. Knowledge of the pathogenesis and host response against EVs and HPeVs is essential to develop effective treatment strategies.

Meanwhile treatment with IVIG, ideally selected for its high nAb titer against the infective EV or HPeV type, is the most feasible option in life-threatening EV and HPeV infections, awaiting the development of monoclonal nAbs. The development of effective antiviral drugs should start with the registration of pleconaril (on compassionate use basis), making this broad spectrum anti-enteroviral drug available for those patients with severe EV infections in whom antibody-based therapy has failed or is likely to fail. The search for other effective drugs, targeting the different aspects of the life cycle of the virus and/or host-related factors should meanwhile be intensified.

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Addendum

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Dutch summary (Nederlandse samenvatting)

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Dutch summary (Nederlandse samenvatting)

Picornavirussen

Picornavirussen zijn veel voorkomende kleine virussen (picorna is grieks voor klein) die een breed scala aan ziektebeelden kunnen veroorzaken. De picornavirussen zijn opgedeeld in 26 genera (geslachten), waarvan 7 genera ook mensen kunnen besmetten. De bekendste zijn *Hepatovirus*, *Enterovirus* en *Parechovirus*. Het *Hepatovirus* genus bevat maar 1 virus type dat besmettelijk is voor mensen, namelijk het hepatitis A virus. Dit virus is de verwekker van hepatitis A oftewel geelzucht. De infectie gaat vanzelf weer over, maar je kan er wel behoorlijk ziek van zijn. Gelukkig is er een effectief vaccin tegen dit virus ontwikkeld.
Het *Enterovirus* genus daarentegen bevat meer dan 200 virus types die mensen kunnen besmetten. Deze zijn weer onderverdeeld in 7 species (soorten); Enterovirus A-D en Rhinovirus A-C. Ziektebeelden veroorzaakt door deze virussen variëren van verkoudheid en diarree tot levensbedreigende infecties van de hartspier of hersenvliesontsteking. Rhinovirussen zijn de meest voorkomende verwekkers van verkoudheid. Een bekend enterovirus is het poliovirus. Dit virus was in het verleden berucht als verwekker van kinderverlamming. Dankzij de ontwikkeling en wereldwijde distributie van een effectief vaccin is dit virus bijna uitgeroeid. In de laatste 20 jaar heeft een epidemie van een ander enterovirus, genaamd enterovirus 71, gezorgd voor talloze ernstige en dodelijke infecties in Azië, met name bij jonge kinderen.
Het *Parechovirus* genus bestaat uit 2 species, het ljunganvirus en het humaan parechovirus. Alleen van het humaan parechovirus is bewezen dat het mensen kan infecteren. Het humaan parechovirus bestaat uit 16 types en kan dezelfde ziektebeelden geven als het enterovirus, maar wordt bijna alleen bij jonge kinderen gezien. Parechovirus type 1 en 3 komen het meest voor, waarbij parechovirus type 1 met name milde klachten van de luchtwegen en het maagdarmstelsel geeft en parechovirus type 3 vaak ernstige infecties, zoals hersen(vlies) ontsteking geeft.

Diagnose

Vroeger werd een virus gediagnosticeerd door middel van een kweek in cellen, omdat virussen altijd een gastheercel nodig hebben om te overleven. Dit kostte veel tijd en was moeilijk, omdat veel virussen slecht groeien. Tegenwoordig zijn er nieuwe (moleculaire) technieken, zoals PCR (Polymerase Chain Reaction), waarbij een deel van het genetisch materiaal waaruit het virus bestaat, wordt vermenigvuldigd en vervolgens wordt afgelezen. Zo kan snel worden aangetoond wat voor soort virus er aanwezig is bij een patient (virus detectie). Door het verder aflezen van de genetische code (d.m.v. sequencing) kan het virus vergeleken worden met genetisch materiaal van reeds bekende virussen (virus typering). Deze nieuwe technieken zijn veel sneller en gevoeliger en hebben ertoe geleid dat er de laatste jaren veel nieuwe virus types zijn ontdekt. Zo is de hele species rhinovirus C ontdekt en bleek parechovirus type 3 vaker voor te komen dan eerder gedacht. Ook werden

rhinovirussen, enterovirussen en parechovirussen veel vaker gevonden, ook bij kinderen zonder klachten.

De rol van antistoffen

Voor de afweer tegen enterovirussen zijn antistoffen van belang. Dit denken we omdat mensen met een ziekte waarbij ze geen antistoffen kunnen maken (primaire immuundeficiënties) erg ziek kunnen worden van enterovirussen. Antistoffen of immuunglobulines worden gemaakt door de B-lymfocyten (een bepaald type witte bloedcel) op het moment dat zij een virus tegenkomen. Deze antistoffen zorgen ervoor dat het virus geneutraliseerd wordt. Ook worden er geheugencellen gemaakt die langdurig antistoffen tegen dat virus laten circuleren. Hierdoor wordt het virus, als het nog een keer het lichaam binnendringt, snel herkend en geneutraliseerd, zodat je er niet meer (zo) ziek van wordt. Tijdens de zwangerschap worden de antistoffen van de moeder via de placenta doorgegeven aan ongeboren baby's, zodat baby's de eerste maanden na de geboorte beschermd zijn door maternale antistoffen. Daarna verdwijnen deze maternale antistoffen en moeten zuigelingen zelf antistoffen gaan maken.

Omdat het parechovirus erg lijkt op het enterovirus denken we dat antistoffen ook van belang zijn voor de afweer tegen deze groep virussen. Dit denken we ook omdat er een verschil in leeftijd is waarop de twee meest voorkomende types, namelijk type 1 en 3, voorkomen. Het parechovirus type 1 komt met name voor bij kinderen ouder dan 6 maanden en geeft over het algemeen milde klachten van een luchtweginfectie of een maagdarminfectie. We weten door eerder onderzoek dat bijna alle volwassenen (92-99%) antistoffen hebben tegen parechovirus type 1. We denken dan ook dat baby's worden beschermd door deze maternale antistoffen en daardoor in de eerste levensmaanden niet ziek worden van dit virus. Het parechovirus type 3 daarentegen komt juist vaak bij pasgeborenen voor en kan ernstige ziektebeelden geven, zoals een hersen(vlies)ontsteking en bloedvergiftiging. Maar heel weinig volwassenen blijken antistoffen tegen dit type te hebben (ongeveer 10-13%). We denken dat de meeste baby's daarom niet beschermd worden door maternale antistoffen en daardoor op zo'n jonge leeftijd ziek kunnen worden van parechovirus type 3.

Behandeling

Er is geen goede behandeling voor ernstige enterovirus en parechovirus infecties. Omdat antistoffen belangrijk lijken te zijn bij de afweer tegen deze virussen, wordt soms een infuus met antistoffen (intraveneuze immunoglobulines) gegeven aan patiënten met een ernstige enterovirus infectie in de hoop dat de antistoffen het virus neutraliseren. Deze intraveneuze immunoglobulines worden gemaakt vanuit een pool van antistoffen van meer dan 1000 donoren. De werkzaamheid hiervan is echter nooit bewezen. Tevens wordt onderzoek gedaan naar antivirale middelen tegen enterovirussen. Tot nu toe is er één middel, genaamd pleconaril, toegepast bij patiënten. Het was bedoeld als middel tegen verkoudheid door rhinovirussen, maar bleek ook effectief tegen andere enterovirussen. Een aantal patiënten

met een ernstige enterovirusinfectie zijn behandeld met pleconaril met wisselend resultaat. Vanwege de bijwerkingen en het risico op resistentie is het middel nooit goedgekeurd als middel tegen verkoudheid en daardoor nooit geregistreerd als medicijn.

Dit proefschrift

Het eerste deel van dit proefschrift gaat over de betekenis van een infectie met rhinovirussen, enterovirussen en parechovirussen bij jonge kinderen. Het tweede deel gaat over de noodzaak voor een behandeling tegen deze virussen en de eventuele mogelijkheden.

In **hoofdstuk 2** hebben we gekeken naar het voorkomen van de verschillende soorten rhinovirussen en de relatie met ernst van klachten in een geboortecohort van Nederlandse kinderen met en zonder luchtwegklachten. Rhinovirus is de meest voorkomende veroorzaker van de gewone verkoudheid. In eerdere studies werd bij jonge kinderen met een verhoogd risico op het krijgen van astma (omdat één van hun ouders astma of allergie had) een relatie gevonden tussen het voorkomen van een piepende ademhaling (wheezing) bij rhinovirus infecties en de ontwikkeling van astma op latere leeftijd. Ook lijkt het recent ontdekte rhinovirus type C geassocieerd te zijn met een piepende ademhaling, astma aanvallen en ernstige infecties zoals een longontsteking. Veel van deze onderzoeken zijn gedaan bij kinderen met een verhoogd risico op astma of bij kinderen opgenomen in het ziekenhuis. In onze studie hebben wij een groep kinderen vanaf de geboorte gedurende enkele jaren gevolgd. Kinderen met luchtwegklachten werden bezocht door een studieteam die de ernst van de klachten evalueerde en een neus- en keelwat afnam om te kijken naar luchtwegvirussen. Dit is ook gedaan bij kinderen uit dezelfde groep zonder luchtwegklachten. Rhinovirus was het meest voorkomende virus bij zowel kinderen met luchtwegklachten (41%) als zonder klachten (26%). Maar liefst 86% van de kinderen met luchtwegklachten bleek besmet te zijn met minimaal één virus en 44% was besmet met twee of meer virussen. Kinderen die alleen met een rhinovirus waren besmet, hadden ernstiger klachten, maar benauwdheid en een piepende ademhaling werd niet vaker gezien bij kinderen met een rhinovirus infectie. Rhinovirus types A en C werden het meest gezien bij kinderen met luchtwegklachten, terwijl rhinovirus type B vaker werd gezien bij kinderen zonder klachten. Er was geen verschil in de ernst van de klachten of het voorkomen van een piepende ademhaling tussen de verschillende types. In tegenstelling tot eerder onderzoek lijkt in deze groep kinderen rhinovirus type C dus niet geassocieerd met ernstiger klachten of een piepende ademhaling. Er is ook gekeken naar de hoeveelheid rhinovirus in neus en keel, waarbij kinderen met luchtwegklachten meer rhinovirus bij zich droegen dan kinderen zonder klachten. Op basis van de hoeveelheid rhinovirus in neus en keel kon er echter geen onderscheid gemaakt worden tussen kinderen met en zonder luchtwegklachten.

Parechovirus infecties worden de laatste jaren steeds vaker gevonden doordat steeds meer laboratoria nieuwe technieken, zoals PCR, gebruiken om deze virussen op te sporen. Het

parechovirus vermenigvuldigt zich in het maagdarmstelsel, waardoor het virus bij een infectie zo goed als altijd in de ontlasting kan worden gevonden. In **hoofdstuk 3** hebben we gekeken naar de klachten van kinderen bij wie een parechovirus type 1 of 3, de meest voorkomende types, in de ontlasting was gevonden tussen 2004 en 2008. Kinderen met een parechovirus type 3 infectie bleken jonger te zijn (gemiddeld 2 maanden oud) en vaker ernstiger infecties, zoals een hersen(vlies)onsteking of bloedvergiftiging te hebben dan kinderen met een parechovirus type 1 infectie (gemiddeld 6 maanden oud). Bij kinderen met een parechovirus type 1 infectie waren de klachten niet altijd gerelateerd aan de infectie, terwijl dat bij een type 3 infectie wel zo goed als altijd het geval was. Bovendien hadden de kinderen, die ziek werden van een parechovirus type 1 infectie, vaker een onderliggende aandoening.

Vervolgens hebben we gekeken naar de duur van de uitscheiding van het parechovirus in de ontlasting na een infectie (**hoofdstuk 4**). De duur van uitscheiding was tussen de 4 en 24 weken en verschilde niet tussen parechovirus types. Over het algemeen hadden de kinderen geen klachten tijdens de periode van virus uitscheiding. De hoeveelheid virus in de ontlasting nam geleidelijk af in de loop van de tijd, maar er was geen minimale hoeveelheid virus waarbij er nog symptomen werden gezien. Opvallend was dat op het moment van diagnose de hoeveelheid virus in de ontlasting minder was bij kinderen met een parechovirus type 3 infectie ten opzichte van de andere types, terwijl bij deze kinderen juist ernstiger symptomen werden gezien.

Omdat enterovirussen en parechovirussen veel in oppervlaktewater voorkomen, hebben we door middel van een vragenlijst gekeken of het gebruik van kraanwater en zwemmen in oppervlaktewater van invloed zijn op het krijgen van een enterovirus of parechovirus infectie bij kinderen onder de leeftijd van 1 jaar (**hoofdstuk 5**). Dit bleek niet het geval te zijn. Wel was de aanwezigheid van oudere broertjes en zusjes een mogelijke risicofactor voor het krijgen van een enterovirus of parechovirus infectie.

In **hoofdstuk 6** wordt de noodzaak voor een behandeling van parechovirus en enterovirus infecties besproken tezamen met de mogelijke opties. Parechovirussen en enterovirussen veroorzaken meestal milde en voorbijgaande symptomen. Echter, deze virussen zijn ook in staat om ernstige klachten te veroorzaken met blijvende restverschijnselen. Momenteel is er geen effectief antiviraal middel tegen enterovirussen en parechovirussen beschikbaar. De ontwikkeling van antivirale middelen concentreert zich op enterovirussen, met name enterovirus 71. Omdat parechovirussen een andere structuur hebben dan enterovirussen, is het niet waarschijnlijk dat de middelen gericht tegen enterovirussen ook zullen werken tegen parechovirussen. Een andere behandeloptie is de toediening van neutraliserende antistoffen. Intraveneuze immunoglobuline (IVIG) bevat antistoffen van meer dan 1000 donoren en wordt soms gebruikt als behandeling voor ernstige enterovirusinfecties. De

uitkomst van deze behandeling varieert, waarbij soms geen effect wordt gezien en soms de patiënt volledig opknapt. De reden voor deze wisselende uitkomsten kan zijn dat de concentratie specifieke antistoffen tegen een bepaald enterovirus of parechovirus type erg kan variëren in IVIG. De concentratie van antistoffen tegen enterovirussen of parechovirussen die veel circuleren in de algehele bevolking zal hoger zijn dan de concentratie van antistoffen tegen enterovirussen of parechovirussen die weinig circuleren. Een oplossing hiervoor zou zijn om type specifieke (monoclonale) antistoffen te ontwikkelen.
Van de tot nu toe ontwikkelde anti-enterovirale middelen is pleconaril het meest bekend. Pleconaril vervormt het kapsel van enterovirussen, zodat ze niet meer kunnen hechten aan de cellen van de gastheer en deze dus niet meer kunnen infecteren. Pleconaril is werkzaam tegen de meeste rhinovirussen en enterovirussen. Het medicijn was in eerste instantie ontwikkeld als middel tegen verkoudheid. Vanwege het risico op bijwerkingen en de mogelijke ontwikkeling van resistentie is het middel echter nooit goedgekeurd door de Food and Drug Authority (FDA), het Amerikaanse college ter beoordeling van geneesmiddelen. Het middel is dan ook nooit voor deze indicatie op de markt gebracht. Ondertussen is pleconaril wel gebruikt als behandeling voor individuele patiënten met ernstige en/of chronische enterovirus infecties met wisselend resultaat. Pleconaril is niet effectief tegen enterovirus 71 en parechovirus infecties.

In **hoofdstuk 7** worden twee patiënten met een agammaglobulinemie (een aangeboren afweerstoornis waarbij de patiënt geen antistoffen kan maken) beschreven die een chronische hersen(vlies)ontsteking hebben, veroorzaakt door een enterovirus. Beide patiënten zijn behandeld met pleconaril. Eén patiënt reageerde goed op de behandeling; de klachten verdwenen volledig, net als het enterovirus. De andere patiënt toonde geen verbetering van de klachten. Het enterovirus was nog altijd aanwezig in het hersenvocht. In het laboratorium is vervolgens getest of het enterovirus dat was gevonden bij de patiënten gevoelig was voor pleconaril. Het enterovirus (echovirus 13) dat was gevonden in het hersenvocht de eerste patiënt was gevoelig voor pleconaril, terwijl het enterovirus (echovirus 11) dat was gevonden in het hersenvocht van de tweede patiënt niet gevoelig was voor pleconaril. Dit toont aan dat pleconaril wel werkzaam is tegen bepaalde enterovirussen en dat door in het laboratorium gevoeligheidstesten te doen, de effectiviteit van de behandeling kan worden voorspeld.

Opvallend was dat echovirus 11 ongevoelig (resistent) was voor pleconaril. In eerdere studies was namelijk aangetoond dat echovirus 11 wel gevoelig is voor pleconaril. Omdat de patiënt nog niet eerder was behandeld met pleconaril, is gekeken of het mechanisme van deze natuurlijke resistentie achterhaald kon worden (**hoofdstuk 8**). Door middel van een driedimensionaal model werden drie verschillen in de genetische code van het virus (mutaties) gevonden op de plek waar pleconaril aan het virus bindt. Eén of meerdere van deze drie mutaties is dus mogelijk de oorzaak van de resistentie. Vervolgens is een pleconaril

A

gevoelige echovirus 11 stam in het laboratorium ongevoelig gemaakt. Bij analyse van de genetische code werden twee andere mutaties gevonden die mogelijk verantwoordelijk waren voor de resistentie. De 5 gevonden mutaties zijn vervolgens geïntroduceerd in een gevoelige echovirus 11 stam. Slechts één van deze mutaties leidde tot een levend én pleconaril ongevoelig virus. De resultaten van deze studie laten zien dat er verschillende mechanismen zijn waardoor een virus resistent kan worden en dat deze mechanismen anders kunnen zijn in patiënten in vergelijking met laboratorium experimenten.

In **hoofdstuk 9** wordt een baby van 4 maanden oud met een ernstige ontsteking van de hartspier beschreven. Bij uitgebreid onderzoek naar de oorzaak werd een parechovirus type 1 in zijn bloed gevonden. De baby had op dat moment geen antistoffen tegen parechovirus type 1. Hij werd behandeld met IVIG en is uiteindelijk volledig opgeknapt. De gebruikte IVIG bleek een hoge concentratie aan parechovirus type 1 antistoffen te bevatten. Na behandeling bevatte het bloed van de patiënt ook een hoge concentratie aan antistoffen. Deze casus laat zien dat behandeling met IVIG effectief kan zijn als IVIG een hoge antistofconcentratie tegen het betreffende enterovirus of parechovirus type bevat.

In **hoofdstuk 10** is gekeken of het verschil in ziektebeelden tussen parechovirus type 1 en type 3 kan worden verklaard door een verschil in voorkeur voor het infecteren van een bepaald type cel (tropisme) tussen de beide parechovirus types. Hiervoor zijn parechovirussen, gevonden bij patiënten met ernstige en minder ernstige klachten, in het laboratorium gekweekt op verschillende celtypes. Met name de parechovirus type 3 stammen die afkomstig waren van kinderen met een hersen(vlies)ontsteking leken beter te groeien in cellen afkomstig van het centraal zenuwstelsel. Tevens is in IVIG batches van verschillende jaren gekeken naar de antistofconcentratie tegen parechovirus type 1 en 3. In alle batches werden hoge antistofconcentraties tegen parechovirus type 1 gevonden en zeer lage antistofconcentraties tegen parechovirus type 3. Bij twee parechovirus type 3 geïnfecteerde donoren is gekeken naar de antistof concentratie ten tijde van de infectie en een jaar later. Op beide momenten waren de antistofconcentraties tegen parechovirus type 3 laag. Hierbij rees de vraag of antistoffen wel van belang zijn bij de afweerreactie tegen parechovirus type 3.

Om de rol van (maternale) antistoffen in de afweerreactie tegen parechovirus infecties bij zuigelingen te evalueren hebben we een case-controle studie opgezet van moeder-kind paren (**hoofdstuk 11**). Kinderen onder de leeftijd van 1 jaar met een bewezen parechovirus infectie en hun moeders werden geïncludeerd als cases. Kinderen met klachten van een virale infectie, maar negatief voor parechovirus, en hun moeders werden geïncludeerd als controles. Vervolgens is de concentratie antistoffen tegen parechovirus type 1, 3 en 4 in bloed van de moeders bepaald. Zo goed als alle moeders (99%) hadden antistoffen tegen parechovirus type 1, terwijl maar 2% van de moeders antistoffen had tegen parechovirus

type 3. Twee derde van de moeders had antistoffen tegen parechovirus type 4. Er was geen verschil in de aan- of afwezigheid of de concentratie aan antistoffen tussen de moeders van de cases en de controles. Zuigelingen besmet met een parechovirus type 1 of 4 bleken duidelijk ouder te zijn (gemiddelde leeftijd 4-5 maanden) dan kinderen besmet met parechovirus type 3 (gemiddelde leeftijd 1 maand). Ernstige infecties werden voornamelijk gezien bij jonge zuigelingen met een parechovirus type 3 infectie.
Omdat bescherming door maternale antistoffen met name van belang is in de eerste levensmaanden, is vervolgens gekeken naar de subgroep van kinderen jonger dan 3 maanden. Bij de moeders van de parechovirus positieve kinderen onder de 3 maanden (voornamelijk type 3) werden geen of lage concentraties antistoffen gevonden tegen het parechovirus dat de infectie bij de baby veroorzaakte. Geen van de met parechovirus besmette kinderen van wie bloed beschikbaar was, had antistoffen tegen het type waarmee ze besmet waren.
De jongere leeftijd waarop kinderen een infectie krijgen met parechovirus type 3 kan verklaard worden door de afwezigheid van beschermende antistoffen tegen dit type. Jonge zuigelingen lijken daarentegen beschermd te worden door maternale antistoffen tegen parechovirus type 1 en 4, aangezien deze infecties alleen gezien worden als maternale antistoffen zijn verdwenen. De vraag blijft wel waarom alleen jonge kinderen zo ziek worden van een parechovirus type 3 infectie als zowel kinderen als volwassenen geen antistoffen hebben tegen parechovirus type 3.

Conclusie

In dit proefschrift beschrijf ik dat infecties met parechovirussen en enterovirussen bij kinderen een breed scala aan symptomen kunnen veroorzaken, variërend van asymptomatisch of milde luchtweg- of maagdarmklachten tot levensbedreigende infecties zoals hersen(vlies) ontsteking en ontsteking van de hartspier. Antistoffen lijken een belangrijke rol te spelen bij de afweerreactie tegen deze virussen, alhoewel dit niet alle verschillen in pathogenese (infectieprocessen) tussen de verschillende enterovirus en parechovirus types kan verklaren. Ondanks dat de meeste infecties met enterovirussen en parechovirussen mild verlopen, laat ik in deze thesis zien dat er wel degelijk behoefte is aan een effectieve behandeling bij ernstige infecties. Op dit moment is er geen antivirale medicatie tegen deze virussen beschikbaar. De beste behandelingsoptie is daarom IVIG, waarbij het effect wel afhankelijk lijkt te zijn van de antistofconcentratie tegen het specifieke enterovirus of parechovirus type. Meer onderzoek naar de afweerresponsen bij de gastheer, de verschillen in infectiviteit tussen de verschillende virustypes en receptorgebruik van deze virussen is nodig om inzicht te krijgen in de pathogenese en om nieuwe behandelingsstrategieën te ontwikkelen.

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List of publications

Wildenbeest JG, van der Schee MP, Hashimoto S, Benschop KSM, Minnaar RP, Sprikkelman AB, Haarman EG, van Aalderen WMC, Sterk PJ, Pajkrt D, Wolthers KC.
Rhinovirus C is not associated with wheezing or severe disease in an unselected birth cohort from the Netherlands. *Submitted.*

Wildenbeest JG, Benschop KSM, Bouma-de Jongh S, Wolthers KC, Pajkrt D. Prolonged shedding of human parechovirus in feces of young children after symptomatic infection. *Submitted.*

Lodder WJ, **Wildenbeest JG**, Bouwknegt M, Rutjes SA, Pajkrt D, Wolthers KC, de Roda Husman AM. Exposure to recreational and drinking water and the occurrence of parechovirus and enterovirus infections in infants. *Submitted.*

Benschop KSM, **Wildenbeest JG**, Koen G, Minnaar RP, van Hemert FJ, Thomas XV, Westerhuis BM, Pajkrt D, van den Broek PJ, Vossen ACTM, Wolthers KC. Genetic and antigenic structural characterization for resistance of Echovirus 11 to pleconaril in an immunocompromised patient. *Accepted in J Gen Virol.*

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Curriculum vitae

Joanne Geraldine Wildenbeest was born on October 30th 1976 in Hengelo, the Netherlands. She grew up in Wageningen and attended secondary school at the Streeklyceum in Ede. In 1995 she started her medical education at the Ghent University in Belgium. During her studies she participated as research student in scientific research at the department of neurology. In 2001 she went to Rio de Janeiro, Brazil, to do scientific research in the field of leprosy. In the same year she went to Durban, South-Africa to do a clinical elective at the pediatric department of the King Edward VIII Hospital, Nelson Mandela School of Medicine. She obtained her medical degree magna cum laude in 2003. Subsequently she started as a resident at the department of pediatrics of the Emma Children's Hospital, AMC (prof. dr. H.S.A. Heymans) and later at the Sint Lucas Andreas hospital (dr. B. Wolf), both in Amsterdam. In 2009 she finished her residency in pediatrics and started her PhD project on enterovirus and parechovirus infections in young children under supervision of dr. D. Pajkrt, dr. K.C. Wolthers, prof. dr. T.W. Kuijpers and prof. dr. M.D. de Jong. In the same period she worked as a pediatrician at the Zuwe Hofpoort hospital (Woerden), the Westfriesgasthuis (Hoorn), the Meander Medisch Centrum (Amersfoort) and the Medisch Centrum Haaglanden (The Hague). In September 2012 she started with a fellowship in pediatric infectious diseases and immunology at the Emma Children's hospital (supervisors prof. dr. T.W. Kuijpers and dr. D. Pajkrt). Since a few years she is also working as a pediatrician at the Sint Lucas Andreas hospital. Joanne lives together with her husband Brian in Amsterdam.

Portfolio		
Name: Joanne G. Wildenbeest		
PhD period: 2009-2014		
Promotores: prof. dr. T.W. Kuijpers en prof. dr. M.D. de Jong		
Copromotores: dr. D. Pajkrt en dr. K.C. Wolthers		
1. PhD training	**Year**	**Workload (ECTS)**
Education and courses		
Practical Biostatistics	2012	1.1
Infectious Diseases	2012	1.3
Basic course in legislation and organisation for clinical researchers (BROK)	2013	0.9
Training Upcoming Leaders in Paediatric Science (TULIPS) PhD curriculum	2011/ 2012	3.0
Infection and Immunity in Children; The Oxford Course. Oxford, United Kingdom	2010	0.9
Vaccination masterclass, Virology education, Utrecht, the Netherlands	2012	0.6
Infection and Immunity in Children; The Oxford Course. Oxford, United Kingdom	2013	0.9
Oral presentations (first author only)		
Duration of shedding of human parechovirus in faeces of young children after symptomatic infection.		
29th Annual meeting of the European Society for Paediatric Infectious Diseases, The Hague, the Netherlands	2011	0.5
33e NVK congres, Veldhoven, the Netherlands	2011	0.5
Absence of neutralizing maternal antibodies against human parechovirus type 3 infection in young children.		
17th Annual Meeting of the European Society for Clinical Virology, Prague, Czech Republic	2014	0.5
Poster presentations (first author only)		
Chronic enterovirus meningoencephalitis in a boy with X-linked agammaglobulinemia; evidence for pleconaril as effective treatment.		
28th Annual meeting of the European Society for Paediatric Infectious Diseases, Nice, France	2010	0.5
32e NVK congres, Veldhoven	2010	0.5
11e EKZ Wetenschapssymposium, Amsterdam	2011	0.5
Duration of shedding of human parechovirus in faeces of young children after symptomatic infection.		
32e NVK congres, Veldhoven	2010	0.5
11e EKZ Wetenschapssymposium, Amsterdam	2011	0.5
1e Amsterdam kindersymposium, Amsterdam	2012	0.5

Comparison of clinical symptoms of HPeV-1 and -3: HPeV 1 is associated with meningitis and sepsis-like illness in young children. *7th World Congress of the World Society for Paediatric Infectious Diseases, Melbourne, Australia*	2011	0.5
Cardiomyopathy due to human parechovirus infection in an infant and effect of treatment with IVIG. *29th Annual meeting of the European Society for Paediatric Infectious Diseases, The Hague, the Netherlands* *1e Amsterdam kindersymposium, Amsterdam*	2011 2012	0.5 0.5
Rhinovirus C infection and association with severity of respiratory symptoms in an unselected pediatric population; the EUROPA-study. *34e NVK congres, Veldhoven*	2012	0.5
(Inter)national conferences, symposia and meetings		
28th Annual meeting of the European Society for Paediatric Infectious Diseases, Nice, France	2010	0.75
29th Annual meeting of the European Society for Paediatric Infectious Diseases, The Hague, the Netherlands	2011	0.75
7th World Congress of the World Society for Paediatric Infectious Diseases, Melbourne, Australia	2011	0.75
1e Amsterdam Kindersymposium, Amsterdam	2012	0.25
Jonge onderzoekersdag, NVK, Veldhoven	2012	0.25
31th Annual meeting of the European Society for Paediatric Infectious Diseases, Milan, Italy	2013	0.75
ID (Infectious Diseases)-week, San Francisco, USA	2013	1.0
DAPS, Bilthoven, the Netherlands	2013	0.25
3e Amsterdam Kindersymposium, Amsterdam	2014	0.25
17th Annual Meeting of the European Society for Clinical Virology, Prague, Czech Republic	2014	0.75
16th Biennial Meeting of the European Society for Immunodeficiencies, Prague, Czech Republic	2014	0.75
2. Teaching		
Lecturing		
SCEM meeting: 'Infecties en de huid, hollen of stilstaan.' Amersfoort, the Netherlands	2012	0.5
Supervising		
Student medicine: Extra-curricular activity as part of the honours program: Paediatric And Adult Causes of Encephalitis and Meningitis (PACEM) study	2011/ 2012	1.0
Student medicine: Extra-curricular activity: Viral Myocarditis in Pediatrics (ViMP) study	2012-2014	1.0
3. Parameters of esteem		
Grants		
Stichting Steun Emma	2012	