Epidemiology and clinical associations of human parechovirus and Ljungan virus

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Abbreviations

ADEM	acute disseminated encephalomyelitis
AOM	acute otitis media
ATCC	American Type Culture Collection
CNS	central nervous system
CPE	cytopathic effect
CSF	cerebrospinal fluid
DIPP study	Diabetes Prediction and Prevention Study
DMEM	Dulbecco’s modified Eagle’s medium
GBS	Guillain-Barré syndrome
EV	enterovirus
IFA	immunofluorescence assay
IVIGs	intravenous immunoglobulins
HPeV	human parechovirus
IRES	internal ribosome entry site
LV	Ljungan virus
MEF	middle ear fluid
MEM	minimum essential medium
nABs	neutralising antibodies
NPA	nasopharyngeal aspirate
PCR	polymerase chain reaction
RT	reverse transcription
RV	rhinovirus
RSV	respiratory syncytial virus
T1D	type 1 diabetes
UTR	untranslated region
VP0-4	viral protein 0-4

Abstract

Human parechovirus (HPeV) and Ljungan virus (LV) are non-enveloped, single-stranded RNA viruses that form the genus *Parechovirus* in the family *Picornaviridae*. The interest in these viruses has notably increased over the past 15 years because of their strengthened associations human and animal diseases. HPeVs include 16 genotypes (HPeV1 to 16) that are globally distributed common pathogens, primarily causing clinically mild or unapparent infections. HPeV types 1 and 3 have also been associated with more severe infections in young children, such as infections of the central nervous system (CNS) and sepsis-like disease. Rodent-infecting LV has been suggested to possess zoonotic potential and to induce various human diseases. However, the proof for this possibility remains lacking. This study aimed to describe the epidemiological features of HPeVs in Finland and in the Netherlands, to examine the connection between HPeV-induced infection and human diseases and to study the circulation of LV in Finland.

The epidemiological analysis of stool samples, which were collected during the period from 1996 to 2007, revealed that HPeVs are highly common in healthy Finnish children. HPeV was primarily detectable in children under 2 years old. Altogether, 39% of the study participants tested positive for HPeV at least once during the study period. HPeVs circulated throughout the year, with a distinct seasonal peak in October-November. The results indicated that not only the previously described HPeV1 but also HPeV genotypes 3 and 6 circulate in Finland.

Microneutralisation assays, which were set up to detect HPeV1 to 6, the most common genotypes in Europe, provided a deeper understanding of HPeV seroprevalence in the Finnish and Dutch populations. Although seropositivity for HPeV1, 2 and HPeV4 to 6 was high and moderate in adults, notably, seropositivity was extremely low for HPeV3. We could attribute this low seroprevalence of HPeV3 to the lack of its neutralisation by antibodies. The serological data demonstrate that HPeV types 1 to 6 might be even more prevalent than previously assumed. All six types of HPeV circulate in Finland.

In addition to HPeV detection in background populations, we presented the first cases of severe infection in neonates with HPeV4 and, subsequently, the first isolation of this genotype in Finland. Five hospitalised neonates with a sepsis-like disease in the fall of 2012 were positive for HPeV. Four of these children had HPeV4, indicating a potential small epidemic of this genotype, whereas one HPeV remained untyped. In addition, we detected HPeV3 in a neonate with suspected viral sepsis in October 2011 and another untyped HPeV in a child with symptoms corresponding to acute disseminated encephalomyelitis in May 2012. Following these findings, we promoted the addition of HPeV detection to routine diagnostics of young children. No connection was observed between HPeVs and the onset of acute otitis media or respiratory infections.

To extend the knowledge regarding other parechoviruses in Finland, we studied LV antibody prevalence in both humans and rodents. The seroprevalence detected for LV was 38% in humans and 18% in bank voles (*Myodes glareolus)*. The observation of LV antibodies in humans is relatively high because LV has never been isolated from humans. These results suggest that an LV or LV-like virus, in addition to HPeVs, circulates frequently among human populations in Finland.

1 Literature review

1.1 Introduction

Together, human parechoviruses (HPeVs) and Ljungan viruses (LVs) form the *Parechovirus* genus in the *Picornaviridae* family. Members of this viral family are characterised by the following shared properties: all members are small, non-enveloped RNA viruses with a 7 to 8.8-kilobase positive-stranded RNA genome encoding a single polypeptide. This family also comprises numerous important human and animal pathogens, such as the species of the well-characterised *Enterovirus* (EV), which is presumably the best-known *Picornaviridae* genus. This genus includes the poliomyelitis-causing poliovirus; herpangina; hand, foot and mouth disease; severe neonatal disease-causing coxsackie- and enterovirus species; and rhinoviruses (RVs), the major cause of the common cold. In contrast to EVs, parechoviruses have primarily been linked to mild or asymptomatic diseases in children since their discovery and, thus, have long been considered clinically irrelevant. However, the publication of HPeV type 3 [Ito et al., 2004], which is associated with sepsis-like disease and with central nervous system (CNS) infections in young children [Harvala et al., 2010], in 2004 has not only raised interest in HPeVs but also has drastically increased their clinical relevance.

1.2 Picornaviridae taxonomy

The *Picornaviridae* family currently consists of 26 genera, which are further divided into 46 species (Table 1). The number of members and the classification within this viral family are constantly evolving due to increasing advances in molecular methods, which allow the rapid discovery of new viruses. The latest update of *Picornaviridae* classification was in March 2014 [Adams et al., 2013; International Committee on the Taxonomy of Viruses (ICTV) website, http://talk.ictvonline.org, accessed August 13, 2014]; however, new updates are to be expected due to the constantly ongoing identification of new viruses that fit the *Picornaviridae* criteria.

In the latest update, several proposed genera were added to the *Picornaviridae* classification scheme [ICTV website]. Additional picornaviruses that have been unassigned thus far are awaiting approval for addition by the ICTV [Knowles et al., 2014; Picornastudygroup]. To further clarify the *Picornaviridae* classification scheme, the picornavirus study group has recently proposed the division of picornaviruses into subfamilies [Knowles et al., 2014]. Recent changes to the present picornavirus classification also include the removal of host names from species names. An example of this update is the renaming of entero- and rhinoviruses, which were formerly known as human enterovirus and human rhinovirus, respectively. The current efforts of the *Picornaviridae* study group suggest a change in the naming of parechoviruses. Members of HPeVs should be called parechoviruses type A, whereas LVs would be designated as parechoviruses type B. Furthermore, this group suggests that Sebokele virus should be introduced to the genus as parechovirus C and that ferret parechovirus should be introduced as parechovirus D [Knowles et al., 2014].

Picornaviruses are further classified into genotypes at the species level. Before the advances in sequencing methods, the typing of picornaviruses was based on the neutralisation of virus isolates by specific antisera; thus, viral types were called serotypes. Serotyping has now been replaced by genetic typing or genotyping, where the sequence of the viral protein 1 (VP1) defines the type. Genotyping has significantly increased the number of identified types, currently accounting for over 450 types.

Table 1 Current members of the *Picornaviridae* and their genotype number, as well as their typical natural host, listed according to the relevant genus. Data collected from Ehrenfeld et al. [2010] and picornaviridae study group homepage (www.picornastudygroup.com, accessed August 13, 2014).

Genus	Species	Number of genotypes	Natural host
Aphthovirus	Foot-and-mouth disease virus	7	+70 species, e.g. cattle, pigs, sheep
	Bovine rhinitis A and B viruses	2 and 1	Cattle
	Equene rhinitis A virus	1	Horses, dromedaries, humans
Aquamavirus	Aquamavirus A	1	Seals
Avihepatovirus	Duck hepatitis A virus	3	Ducks
Avisivirus	Avisivirus A	1	Turkeys
Cardiovirus	Enchephalomyocarditis virus	2	+30 species, including mammals, birds, and vertebrates
	Theilovirus	12	Mice, rats, humans
Cosavirus	Cosavirus A	24	Humans
Dicipivirus	Cadicivirus A	1	Dogs
Enterovirus	Enterovirus A-J	Altogether 145	E.g. humans, monkeys, pigs, cattle
	Rhinovirus A-C	Altogether 166	Humans
Erbovirus	Equine rhinitis B virus	3	Horses
Gallivirus	Gallivirus A	1	Turkeys, chickens
Hepatovirus	Hepatitis A virus	1	Humans, monkeys
Hunnivirus	Hunnivirus A	3	Cattle, sheeps
Kobuvirus	Aichivirus A, B and C	3, 2 and 1	Humans
Megrivirus	Melegrivirus A	1	Turkeys
Mischivirus	Mischivirus A	1	Bats
Mosavirus	Mosavirus A	1	Mice
Oscivirus	Oscivirus A	2	Wild birds
Parechovirus	Human parechovirus	16	Humans, monkeys
	Ljungan virus	4	Rodents, humans?
Pasivirus	Pasivirus A	1	Pigs
Passerivirus	Passerivirus A	1	Wild birds
Rosavirus	Rosavirus A	1	Mice
Salivirus	Salivirus A	1	Humans
Sapelovirus	Porcine sapelovirus	1	Pigs
	Simian sapelovirus	3	Monkeys
	Avian sapelovirus	1	Ducks
Senecavirus	Seneca valley virus	1	Pigs
Teschovirus	Porcine teschovirus	13	Pigs
Tremovirus	Avian encephalomyelitis virus	1	E.g. chicken, turkeys, pheasants
Unassigned species	Several species	+28	Seals, ticks, humans, bats, snakes, rodents and at least four bird and eight fish species

1.2.1 The *Parechovirus* genus

The first two parechoviruses discovered were originally classified as EVs and named echovirus 22 and 23 [Wigand and Sabin, 1961]. In 1999, parechoviruses were separated from EVs based on molecular, biological and functional differences [Hyypia et al., 1992; King et al., 2000] to form their own genus. Subsequently, echovirus 22 and 23 were renamed human parechovirus 1 and 2, respectively. Shortly after its formation, this genus was joined by another species, namely, Ljungan virus, which is a suspected zoonotic virus isolated from a bank vole [Niklasson et al., 1999]. Advances in molecular virus discovery and in reverse transcription (RT) polymerase chain reaction (PCR) technology have allowed the identification of new parechoviruses. Because LVs were discovered from samples collected in the 1960s and 1980s, parechoviruses appear to have remained undetected due to a lack of sufficient technology. Presumably, their lack of frequent association with severe clinical cases also delayed their discovery. Thus far, 4 LV and 16 HPeV genotypes have been described (Table 2). As aforementioned, two additional viral species, the rodent-borne Sebokele virus [Joffret et al., 2013] and a ferret parechovirus [Smits et al., 2013], have recently been suggested to join the *Parechovirus* genus.

Although humans are the primary hosts of HPeVs, some HPeV types have also been discovered in synanthropic non-human primates (NHPs). These NHP species include mandrills (*Mandrillus sphinx*) and pigtail macaques (*Macaca nemestrina*) not living in the wild [Oberste et al., 2013a], as well as rhesus macaques (*Macaca mulatta*) living in close contact with human populations [Oberste et al., 2013b]. Instead of humans, the original detection of HPeV12 and HPeV15 was in rhesus macaques. In addition to these findings, HPeV genotypes 1, 4, 5, 6 and 14 have also been detected in macaques [Oberste et al., 2013b; Shan et al., 2010].

Table 2 Parechovirus genotypes discovered to date, listed according to year, place, source, and reference of discovery.

Genotype	Sampling year	Place	Source	Reference
HPeV1	1956	USA	Feces from children with diarrhea	Wigand and Sabin, 1961
HPeV2	1956	USA	Feces from children with diarrhea	Wigand and Sabin, 1961
HPeV3	1999	Japan	Feces of a 1-year-old child with transient paralysis, fever and diarrhea	Ito et al., 2004
HPeV4	2002	The Netherlands	Feces from an 8-week-old child with fever	Benschop et al., 2006a
HPeV5	1986	USA	Originally classified as HPeV2, feces of a 2-year-old with high fever	Oberste et al., 1998
HPeV6	2000	Japan	Cerebrospinal fluid from a 1-year-old with Reye's syndrome	Watanabe et al., 2007
HPeV7	2007	Pakistan	Feces from a healthy 2-year-old	Li et al., 2009
HPeV8	2006	Brazil	Child with enteritis	Drexler et al., 2009
HPeV9	2004	Bangladesh	Human feces	Nix et al., 2013 Oberste et al., 2013b
HPeV10	2005	Sri Lanka	Feces from a child with gastroenteritis	Kim Pham et al., 2010
HPeV11	2005	Sri Lanka	Feces from a child with gastroenteritis	Pham et al., 2011
HPeV12	2004	Bangladesh	Feces from rhesus macaque	Nix et al., 2013 Oberste et al., 2013b
HPeV13	2005	Bangladesh	Human feces	Oberste et al., 2013b
HPeV14	2004	The Netherlands	Human feces	Benschop et al., 2008b
HPeV15	2008	Bangladesh	Feces from rhesus macaque	Oberste et al., 2013b
HPeV16	2008	Bangladesh	Human feces	Oberste et al., 2013b
LV1	1987	Sweden	Bank vole	Niklasson et al., 1999
LV2	1987	Sweden	Bank vole	Niklasson et al., 1999
LV3	1962	USA	Montane vole	Johansson et al., 2003
LV4	1964	USA	Red-backed vole	Tolf et al., 2009

According to recent estimations, all HPeVs presently in circulation share a common ancestor that dates back approximately 400 years [Faria et al., 2009]. From this single ancestor, different lineages are thought to have evolved to form all present genotypes. Typical for RNA viruses, the mutation rate of HPeVs is high [Drake and Holland, 1999; Faria et al., 2009], enabling their rapid evolution. Recombination, which occurs among existing genotypes and which greatly affects their genomic composition, aggravates further the genotype evolution [Benschop et al., 2008c; Sun et al., 2012]. Recombination events cause much larger changes to the HPeV genome than single point mutations. Thus, the true diversity of the present, constantly evolving HPeV population in humans may be far greater than the fraction detected by VP1 sequencing [Baumgarte et al., 2008].

1.3 Virus structure and genome

Similar to other picornaviruses, parechoviruses are non-enveloped, positive-sense single-stranded RNA viruses. The virion of parechoviruses measures approximately 22-30 nm in diameter and has icosahedral symmetry. Their genome is an approximately 7.3 kilobase RNA molecule with a cap protein, Vpg, at its 5'-end and with a polyadenylation site at its 3'-end. This molecule consists of untranslated regions at the 5'- and 3'-ends preceding and following a single open reading frame encoding for a polyprotein (Figure 1). The untranslated region at the 5'-end forms secondary structures, which include a type II internal ribosome entry site (IRES) [Nateri et al., 2000; Nateri et al., 2002]. This genome acts as an mRNA for IRES-mediated translation, which produces a polyprotein containing all of the viral proteins. Three structural proteins, which are designated viral protein 0 (VP0), 1 (VP1) and 3 (VP3), are at the 5'-end of this polyprotein. A combination of these structural proteins aligns to a unit that forms the core structure of the viral capsid. The final icosahedral capsid represents an assembly of 60 copies of this core unit.

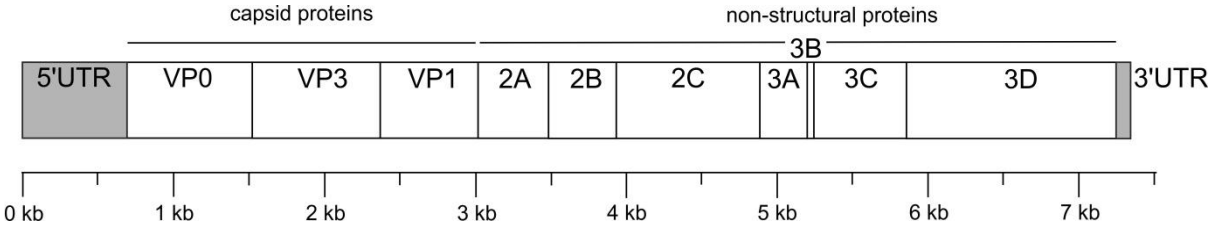

Figure 1 Organisation of the parechovirus genome. The function of each gene according to current knowledge is presented in the text. Grey boxes at the ends of the genome denote the two untranslated regions (UTRs).

VP0, VP1 and VP3 are succeeded in the polyprotein by non-structural 2A-2C and 3A-3D proteins. Of these proteins, the 2C protein has NTPase activity and participates in replication complex formation [Krogerus et al., 2003; Samuilova et al., 2006], the 3B protein (Vpg) acts as the primer for RNA replication, and the 3C protein functions as a protease in polyprotein cleavage [Schultheiss et al., 1995]. The 3D protein is an RNA-dependent RNA polymerase, which executes genome replication. The exact functions of the 2A, 2B and 3A proteins have yet to be examined in detail, although the 2A protein has a suggested function in viral replication [Samuilova et al., 2004], and the 2B protein has a suggested function in membrane permeability [Stanway et al., 2000].

1.4 Replication strategy

The primary replication strategy of parechoviruses closely resembles that of other picornaviruses (Figure 2). Although the current knowledge is heavily based on picornavirus studies, certain steps of the replication cycle have specifically been characterised for HPeV1. The recognition of target receptors on the cell membrane of the host initiates the parechovirus life cycle. HPeV1 binds to $\alpha_v\beta$ integrins [Seitsonen et al., 2010; Triantafilou et al., 2000] using an arginine-glycine-aspartic acid (RGD) motif in the C-terminus of its VP1 [Stanway et al., 1994]. However, this motif is only present in some of the HPeV genotypes. HPeV3 and many of the recently discovered HPeV types lack the RGD motif, and their receptor route remains unknown. HPeV1 studies have demonstrated that parechoviruses enter the host cell through a clathrin-

mediated endolytic pathway after binding to receptors [Joki-Korpela et al., 2001]. Following internalisation, the parechovirus viral genome is freed into the cytosol, where the genome reaches the host cell ribosomes and adopts the role of mRNA in IRES-mediated translation.

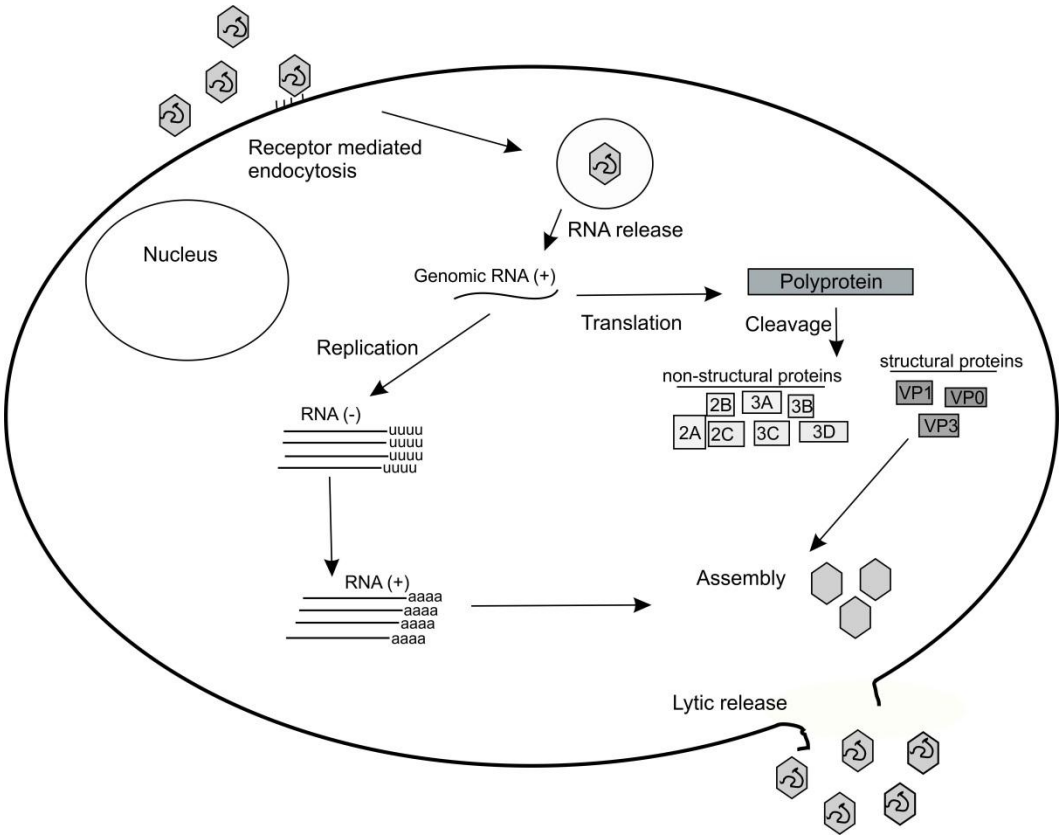

Figure 2 The suggested lytic life cycle for parechoviruses. Host cell recognition initiates the virus life cycle and launches viral entry through endocytosis. Viral RNA is released to the cytosol, where this RNA is first translated to produce viral proteins, which participate in replicating the genome and forming viral capsids. Newly produced viral RNA is packed into the capsid before lytic release from the host cell.

The viral polyprotein is produced by translation and is proteolytically cleaved by the 3C protein to produce viral proteins. After all viral proteins have been produced, the life cycle shifts to RNA replication. This replication process is governed by the viral peptide 3D. First, the positive 3D replicates RNA strand into a complementary negative-strand RNA, which consequently serves as a template for new viral

genomes. HPeV RNA replication occurs in vesicular structures. For HPeV1, the replication complex is thought to be built from Golgi-related vesicles, whose formation is thought to be mediated by the viral peptide 3D [Krogerus et al., 2003].

The newly produced viral molecules, structural proteins and 5'-end Vpg-capped positive-strand RNAs assemble to form new virus particles. These newly born viral particles still undergo a maturation step before becoming infective. The mechanism behind the maturation process in parechoviruses remains unknown. However, this mechanism differs from that of most picornaviruses because cleavage of the VP0 protein into VP2 and VP4 proteins is absent in HPeVs [Stanway et al., 2000]. In the lytic life cycle of picornaviruses, the newly built virions are released after host cell lysis [Cann, 2012]. The majority of picornaviruses disrupt the translation of host cell proteins to enhance the production of the viral polyprotein during an ongoing infection. The life cycle of parechoviruses, lack this step [Coller et al., 1990; Stanway et al., 2000].

1.5 Epidemiological aspects of HPeV

HPeVs are globally distributed and have been detected on all populated continents. Most studies regarding these viruses are of clinical nature, thus primarily linking HPeVs to human diseases. The global distribution varies between HPeV types. Although HPeV types 1-6 have been detected globally, the circulation of other types appears to be more restricted. Fewer reports involve HPeV genotypes 7-16. Thus far, HPeV7, 8, 9 and 12 have been detected in South America and Asia in children with gastrointestinal disease or without a known clinical condition [Alam et al., 2013; Alam et al., 2012; Drexler et al., 2009; Li et al., 2009; Nix et al., 2013; Oberste et al., 2013b; Zhang et al., 2011; Zhong et al., 2011]. Genotypes 10, 11, 13, 14 and 15 have been detected in samples from gastroenteritic or asymptomatic children and from monkeys in Asia [Alam et al., 2013; Kim Pham et al., 2010; Oberste et al., 2013b; Pham et al., 2011].

Currently, the original HPeV14 finding from a stool sample of a Dutch child is the only report of this type in Europe [Benschop et al., 2008b]. Genotype 16 was recently identified from a human stool sample in Bangladesh [Nix et al., 2013; Oberste et al., 2013b].

Multiple HPeV genotypes simultaneously circulate among human populations. Surveillance data of sewage samples from Scotland and from the Netherlands indicate a high presence of HPeVs in the environment, with HPeV3, 6 and 1 being the most common genotypes [Harvala et al., 2014; Lodder et al., 2013]. Serological data of HPeV1 from Canada and from Finland suggest that most individuals (from 72 to over 90%) experience their first HPeV1 infection before two years old [Abed et al., 2007; Tauriainen et al., 2007]. The seropositivity for HPeV1 increases to over 90% in the adult population, whereas the seropositivity for HPeV3 remains slightly lower, at 87% [Ito et al., 2004; Joki-Korpela and Hyypia, 1998; Tauriainen et al., 2007]. Because HPeV1 and HPeV3 are predominantly found in children under the age of three years, HPeVs are thought to primarily target children [Grist et al., 1978; Harvala and Simmonds, 2009; Khetsuriani et al., 2006]. According to studies conducted between 1983 and 2005 in the USA, most HPeV1 (73%) and HPeV2 (68%) infections occur in children under one year old [Khetsuriani et al., 2006]. This observation that HPeVs target young children has been widely confirmed [Benschop et al., 2006b].

Although primarily reported in young children, a few reports have described HPeV infections in adults. In Japan, an HPeV3 epidemic resulted in a series of myalgia cases in adults over 30 years old [Mizuta et al., 2012]. In addition to Japan, sporadic HPeV findings in adults have also been reported for Canada and for Jamaica [Abed and Boivin, 2006; Figueroa et al., 1989; Watanabe et al., 2007]. The concentration of HPeV findings in children differs from that of EVs, which tend to affect individuals of all ages.

1.5.1 Seasonality of HPeV circulation

HPeV infections have been observed to occur throughout the year. The peak of infections varies among geographical areas and genotypes. Although the detection rate of HPeV1 has been reported to be the highest during the period from September to December [Tapia et al., 2008], the detection rate of HPeV3s peaks during summer months [Harvala et al., 2011; Schuffenecker et al., 2012]. Studies from Scotland and from the Netherlands have reported a biennial cycle for HPeV3 infections [Benschop et al., 2008b; Harvala et al., 2011], with a high frequency of infections in even-numbered years and with a lack of the virus in odd-numbered years. This cycle, though was absent in a more recent study from Denmark [Fischer et al., 2014].

1.6 Clinical features of HPeV infection

Similar to other members of *Picornaviridae*, HPeVs primarily replicate in the intestine and are therefore transmitted via the faecal-oral route. However, replication appears to also occur in the respiratory tract, and HPeV has been detected in respiratory secretions [Harvala and Simmonds, 2009]. Because HPeV is predominantly shed in the stool, this virus is detectable in faecal samples. HPeV may also enter the blood stream [Noordhoek et al., 2008; Pineiro et al., 2010, Shoji et al., 2013], thereby spreading to and affecting other organs. A typical HPeV infection presents itself similar to an EV infection. The clinical course and outcome of HPeV infection are often mild or asymptomatic. The age of the child and the HPeV genotype are crucial factors influencing the severity of the infection course and, hence, the outcome [Wildenbeest et al., 2014]. Severe cases concentrate on young (less than 6 months old) children with HPeV type 3 infections.

HPeVs were originally described in children suffering from summer diarrhoea [Wigand and Sabin, 1961]. Since their initial detection, numerous studies have been conducted, reporting their association to this disease and to several other types of gastroenteritides. In fact, most HPeV types have been identified in children suffering from gastroenteritis [Alam et al., 2013; Pham et al., 2011; Zhang et al., 2011].

HPeVs have also been associated with respiratory tract infections in an increasing number of studies [Harvala et al., 2008; Khetsuriani et al., 2006; Pajkrt et al., 2009; Sharp et al., 2012a]. However, the definite causative relation between HPeVs and gastrointestinal or respiratory tract diseases remains to be established. The typical outcome of diseases associated with HPeV in either area is mild. Of all HPeVs, HPeV1, which has also been associated with acute otitis media (AOM) [Tauriainen et al., 2008], is the most frequently detected type in HPeV-induced infections.

In addition to these relatively common observations, single reports of sporadic cases have associated HPeVs with several other diseases, including Guillan-Barré syndrome [Linden et al., 2012], myocarditis [Russell and Bell, 1970], necrotising enterocolitis [Birenbaum et al., 1997], haemolytic uremic syndrome [Oregan et al., 1980], myositis, Reye's syndrome and lymphadenitis [Watanabe et al., 2007]. HPeV types 1, 3 and 6 have also been detected in sporadic cases of acute flaccid paralysis in children [Figueroa et al., 1989; Ito et al., 2004; Watanabe et al., 2007].

HPeV type 4 was originally isolated from a Dutch neonate with fever in 2002 [Benschop et al., 2006]. Since then, HPeV4 has been detected in samples from asymptomatic children and from children with gastrointestinal or respiratory symptoms [Boros et al., 2010; Chen et al., 2009; Pajkrt et al., 2009; Zhang et al., 2011; Zhong et al., 2011]. Based on single cases, a few studies have also proposed that HPeV4 is associated with TORCH syndrome [Schnurr et al., 1996] and with lymphadenitis [Watanabe et al., 2007]. However, the association of HPeV4 with any specific illness has remained to be established.

1.6.1 Neonatal infections

HPeV3 may cause CNS infections and sepsis-like disease in neonates [Harvala et al., 2010]. The discovery of this genotype has drastically increased the interest in and the clinical relevance of HPeVs because HPeV infections have previously been primarily linked to mild clinical presentations. In the years after the first report of HPeV3, particularly over the past six years, reports regarding severe HPeV infections with CNS involvement or with sepsis-like disease have significantly grown in number. Recently, HPeV3 was even detected as the most prevalent picornavirus genotype in young children with CNS-related diseases [Harvala et al., 2011]. Before HPeV3, only HPeV1 was linked to infections with a more severe clinical outcome, such as paralysis or encephalitis [Figueroa et al., 1989; Koskiniemi et al., 1989]. Interestingly, all of the recent studies regarding severe HPeV infections, except for a single report by Zhong et al. [2013], have solely involved HPeV3. All these studies substantiate the important role of HPeV3 as a causative agent of severe infections that have occurred in several European areas and in areas of the USA, Israel, China and Korea (Table 3).

HPeV3-induced CNS-related diseases include viral meningitis [Wolthers et al., 2007; Wolthers et al., 2008] and meningoencephalitis [Verboon-Maciolek et al., 2008a; Verboon-Maciolek et al., 2008b]. Some studies have also linked HPeV3 to white matter damage in neonates, and neuronal HPeV infection in young children may lead to aberrations in the white matter [Belcastro et al., 2014; Gupta et al., 2010; Verboon-Maciolek et al., 2008a]. Some of these children presented neurodevelopmental effects later, including learning disabilities and the development of post-natal epilepsy [Verboon-Maciolek et al., 2008a]. Further studies with a larger study population and with a longer follow-up are still required to determine the long-term effects of these infections.

Table 3 Recent studies on HPeV findings in children with severe diseases, listed according to place and date conducted. Clinical presentation and patient age are indicated in connection to HPeV prevalence and type detected.

Country / Author	Year(s)	Clinical presentation	HPeV prevalence*	Types	Patient age
the Netherlands / Wolthers et al. [2008]	2004 to 2006	CNS-related or sepsis-like disease	33/716, 4.6%	unknown	< 5 years
Scotland / Harvala et al. [2009]	2006 to 2008	sepsis-like disease	14/1575, 0.9%	14 HPeV3s	<3 months
Spain / Pineiro et al. [2010]	2006 to 2009	febrile illness, sepsis-like disease	9/397, 2.3%	8 HPeV3s	>7 months
Scotland / Harvala et al. [2011]	2005 to 2010	CNS-related disease	31/4168, 0.7%	30 HPeV3s	<3 months
France / Schuffenecker et al. [2012]	2008 to 2010	sepsis-like disease	33/1128, 3%	28 HPeV3s, 1 HPeV4	<6 months
France / Mirand et al. [2012]	2010	sepsis-like disease	4/100, 4%	4 HPeV3s	<4 months
USA / Selvarangan et al. [2011]	2006 to 2008	sepsis-like disease	58/780, 7%	52 HPEV3s, 1 HPeV1	0-7 months, mean 1.5 m.
USA / Renaud et al. [2011]	2009 to 2010	CNS-related or sepsis-like disease	15/499, 3.4%	11 HPeV3s	<3months
USA / Walters et al. [2011]	2005 to 2010	CNS-related or sepsis-like disease	10/421, 2.4%	10 HPeV3s	0-2 months
USA / Sharp et al. [2012b]	2009	sepsis-like disease	66/388, 17%	51 HPeV3s	<5 months
Israel / Ghanem-Zoubi et al. [2013]	2007 to 2009	CNS-related or sepsis-like disease	13/367, 3.5%	13 HPeV3s	<3months
China / Zhong et al. [2013]	2008 to 2011	CNS-related disease	68/776, 8.8%	28 HPeV1s, 3 HPeV3s	0-13y, median 14m.
South-Korea / Han et al. [2013]	2011 to 2012	CNS-related or sepsis-like disease	12 /183, 6.5%	HPeV3	<5 months

CNS-related disease includes meningitis and encephalitis.

*HPeV prevalence in cerebrospinal fluid (CSF) samples collected from children with suspected central nervous system infection.

A typical neonatal HPeV infection presents with high fever and irritability. Additionally, the patient may have seizures, a rash and apnoea. In contrast, sepsis-like disease consists of fever or hypothermia, with respiratory dysfunction measured by tachycardia or bradycardia, low blood pressure and decreased oxygen saturation [Wolthers et al., 2008]. In connection with an HPeV infection, the term sepsis-like disease is often used when the patient presents with some sepsis-like symptoms, although not fulfilling all of the aforementioned criteria. The severity of neonatal sepsis may vary greatly between a mild febrile illness and a potentially extremely severe systemic infection with CNS involvement [Harvala et al., 2010]. Compared with EVs, neonatal HPeV infections typically contain lower peripheral white blood cell counts, higher maximum temperatures, longer fever duration, pleocytosis absence, and longer patient hospitalisation [Felsenstein et al., 2013; Renaud et al., 2011; Sharp et al., 2012b].

Despite their severity, HPeV infections rarely have lethal outcomes. In an EV surveillance report conducted in the USA between the years 1983 and 2005, ten lethal cases of HPeV1 infection were reported [Khetsuriani et al., 2006]. In another study, HPeV types 1, 3 and 6 were detectable in postmortem specimens although with no apparent connection to the death of the patients [Sedmak et al., 2010]. HPeV3 has also been reported as a causative agent in sporadic fatal cases of neonates in Denmark [Fischer et al., 2014], France [Schuffenecker et al., 2012] and the Netherlands [van Zwol et al., 2009].

1.6.2 Diagnostic assays

Traditional EV detection with targeted, monoclonal antibody neutralisation detected the first parechoviruses, HPeV1 and HPeV2. Because of this method, HPeVs were long diagnostically considered part of EVs. These initial detection methods were based on viral propagation in cell cultures. Due to their laborious, time-consuming and strain-dependent nature, cell cultures are now widely replaced by real-time RT-PCR methods.

Because of the genetic differences between HPeV and EV, HPeV remains undetected with EV RT-PCR. Thus, the transition to RT-PCR methods led to a complete lack of HPeV detection in many laboratories.

HPeV real-time RT-PCR targets a highly conserved region in the 5'-end of the parechovirus RNA genome. This method is rapid, specific, and sensitive because viral RNA is detectable in a broad array of samples, including faecal, blood, cerebrospinal fluid (CSF), and different respiratory tract samples [de Crom et al., 2013]. During the acute phase of a severe neonate infection with sepsis-like syndrome or with meningitis, HPeV may be directly detected from blood [Wildenbeest et al., 2013] and CSF samples [Harvala et al., 2009]. The addition of HPeV detection into routine diagnostics has frequently been requested [Baumgarte et al., 2008; Pham et al., 2011; Sharp et al., 2012b; Zhong et al., 2013]. Hence, several multiplex RT-PCR methods, including HPeV detection, have been developed in recent years [Jokela et al., 2005; Katano et al., 2011; Noordhoek et al., 2008; Pham et al., 2010]. Picornavirus genotyping is performed using RT-PCR targeted to a more variable VP1 region [Harvala et al., 2008; Nix et al., 2008].

In contrast, the detection of parechovirus-specific antibodies is challenging because of limitations in the type specificity. Thus far, neutralisation tests set up for specific genotypes have been used to study the seroprevalence of HPeV1 and HPeV3. However, these tests are time-consuming, slow and laborious compared with other serological methods. Setting up more efficient methods has proven challenging due to cross-reactions between genotypes. An ELISA-based method for the detection of HPeV1 [Yu et al., 2012] represents the most recent advance. Unfortunately, the risk of cross-reaction is also extremely high for this method. The fact that practically all humans have antibodies against HPeV1 after the first years of life further complicates the development of this methodology.

1.6.3 Treatment and prevention

Currently, no specific antiviral treatment against HPeV infection is available. Therefore, the treatment of neonatal HPeV-induced infection mainly includes supportive measures because the primary focus lies on treating the symptoms. However, a recent report described a case of HPeV1-induced myocarditis, where treatment with intravenous immunoglobulins (IVIGs) was successful [Wildenbeest et al., 2013]. This report further suggested that IVIGs are likely to include neutralising antibodies against viruses with high prevalence, thus benefiting the treatment of a patient. Previously, IVIGs have been used in neonatal EV infections [Abzug et al., 1995]. Another suggested option for treating HPeV infection is the use of human monoclonal antibodies [Wildenbeest et al., 2010], which have been successfully used against influenza virus [Friesen et al., 2010] and respiratory syncytial virus (RSV) [Kwakkenbos et al., 2010]. However, for HPeV this treatment method remains to be developed. Preventing HPeV infections is based on common practices of good hygiene, which are also recommended for other gastrointestinal pathogens. No vaccine against HPeV is currently available.

1.7 Epidemiological features of LV

Ljungan virus was first isolated from bank voles (*Myodes glareolus*), home to the Ljungan Valley in Sweden, during a search for infectious agents associated with a myocarditis epidemic in humans [Niklasson et al., 1999]. Since that initial discovery, this virus has also been detected in other vole species (*Microtus montanus* and *Myodes gapperi*) [Johansson et al., 2003; Tolf et al., 2009] in yellow-necked mice (*Apodemus flavicollis*) [Hauffe et al., 2010], and in Eurasian red squirrels (*Sciurus vulgaris*) [Romeo et al., 2014]. Recently, a potentially new LV type was detected in a faecal sample from an urban rhesus macaque [Oberste et al., 2013b]. LV-specific antibodies have also been reported from other rodent species, such as field voles (*Microtus agrestis*) [Forbes et al., 2014].

Interest in LV has increased due to its suggested zoonotic potential as a causative agent of severe human diseases, including type 1 diabetes (T1D), myocarditis, encephalitis, Guillain-Barré syndrome (GBS), sudden infant death syndrome [Niklasson et al., 2009b] and intrauterine foetal death [Niklasson et al., 2009a; Niklasson et al., 2007]. However, establishing a clear connection between LV and human diseases has proven to be difficult. The cyclical peaks in bank vole population densities every three to four years [Hansson and Henttonen, 1985] correlate with the incidence of T1D, myocarditis and GBS in humans [Niklasson et al., 1998]. LV has been suggested to be the link explaining this correlation. LV infection has been demonstrated to cause T1D, myocarditis and encephalitis [Niklasson et al., 2006], as well as perinatal death [Samsioe et al., 2008], in bank voles, experimental mice and lemmings (*Lemmus lemmus*).

The connection between LV and these diseases in humans remains to be confirmed. The association of LV infection with human cases of intrauterine foetal death, malformations and placental inflammation [Niklasson et al., 2007; Samsioe et al., 2009] is controversial [Krous and Langlois, 2010] and requires further evidence. LV detection in human samples using RT-PCR methods has been described in a few cases, whereas virus isolation has been unsuccessful thus far [Niklasson et al., 2009b; Niklasson et al., 2007; Samsioe et al., 2009; Tapia et al., 2010].

2 Study aims

The primary goal of this project was to obtain a deeper understanding of the epidemiological behaviour of parechoviruses and to more closely examine their clinical associations. The specific aims were to investigate the following:

- the prevalence and epidemiological features of HPeV in a healthy Finnish population (I),
- the seroprevalence of HPeV in different age groups of Finnish and Dutch populations (II),
- the connection of HPeV to severe infections in Finnish infants (III, IV),
- the role of HPeV infection in respiratory tract diseases and in acute otitis media (IV), and
- the presence of antibodies against Ljungan virus in Finnish human and rodent populations (V).

3 Materials and Methods

3.1 Materials

3.1.1 Ethical approvals

Ethical approvals were separately required for human specimens from each study group. The ethical approval for collecting and using specimens from the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study participants was obtained from the Ethics Committees of Tampere and Turku University Hospitals (permission no. 97193M, R12036 and 10/1994). The Ethics Committee of the Tampere University Hospital also approved the study of samples from respiratory infection patients (permission no. R07211). The samples derived from the Finnish Otitis Media Vaccine Trial were collected with permission from the ethical review committees of the National Institute of Health and Welfare, Helsinki University Hospital (permission no. 28/13/03/00/2012) and from the relevant municipal health care authorities. Informed consents were obtained from the individuals or from the parents of the children enrolled in each of these studies. The Ethics Committee of the Helsinki University Hospital approved the study of samples from hospitalised children with unknown infections (permission no. TYH2014251) and the study of human specimens for Ljungan virus (permission no. TYH2013357). The Dutch serum samples were collected from patients visiting the Academic Medical Center (AMC) in Amsterdam. The use of patient sera obtained for diagnostic purposes has been approved under the Research Code of the AMC.

Capturing rodents using techniques such as live- and snap-trapping are not considered an animal experiment according to the Finnish Act on the Use of Animals for Experimental Purposes (62/2006) and the Finnish Animal Experiment Board's decision (May 16th, 2007). Thus, no ethics approval from the Finnish Animal Experiment Board was required for this study.

3.1.2 Serum and faecal specimens from DIPP study participants (I and II)

A summary of all sample materials is presented in Table 4. DIPP study concentrates on studying various aspects of the beta-cell destruction process leading to T1D, including the environmental factors that are associated with an increased risk for T1D. The children are recruited to this ongoing follow-up study according to their HLA-defined increased genetic risk of developing T1D [Ilonen et al., 1996; Kupila et al., 2001]. The children enrol in the study at three months old; first, these children visit the study clinic every three months and, later, every six to 12 months. Blood samples are drawn at each visit and are tested for T1D-associated autoantibodies. The parents are instructed to collect faecal samples monthly and to deliver these samples through regular mail to the laboratory. Additionally, parents complete questionnaires regarding different aspects that might be connected to T1D, such as breast-feeding, the number of siblings, the time of starting day care, pets, infectious diseases, other health issues, etc. Some children are more closely monitored for diet, and different interventions have been tested, for example, different milk formulas during the first few months of a child's life. Since the beginning of the DIPP study in 1994 to present, over 150000 children have been screened for genetic T1D risk at birth, 8500 children have been recruited to the follow-up study, and over 300 children have developed the disease [DIPP studygroup homepage, accessed the 15th of May 2014].

Altogether, 2236 faecal samples, which were collected between the years 1996 and 2007 in Turku and Tampere from 200 children aged 3-72 months, were selected for analysis. Most of the samples were from children younger than 24 months. Fifty-six children were considered cases (2 or more T1D-risk autoantibodies) and the rest of the children were considered controls, which were matched according to gender, HLA-defined risk for T1D, place and time of birth. A set of serum samples from children in age groups of 1, 5 and 10 years, with 144-149 samples per group, were selected for HPeV antibody analysis.

Sixty-one children had provided a serum sample for each time-point, whereas 68 children had provided two samples, and 121 children provided a single sample, for

440 serum samples from 250 children. Seven additional serum samples were selected from children aged 2 years for the analysis of HPeV detection efficiency in stool samples.

Table 4 Sample sets and groups used for detection of HPeV or antibodies against HPeV or LV

Group name	Age range	Subjects	Samples	Sampling time	Location	Method	Study
DIPP-participants	3-72 months	200	2236 stool	1996-2007	Tampere, Turku	HPeV RT-PCR	I
DIPP-participants	1, 2, 5 and 10y	257	447 serum	1994-2010	Tampere, Turku	HPEV microneutralization	I, II
Dutch patients	1-5y, 20-30y, 40-60y	114	114 serum	2010-2011	Amsterdam, the Netherlands	HPEV microneutralization	II
Medical students	20-30y	72	72 serum	2008-2009	Tampere	HPEV microneutralization	II
AOM-patients	2.7-25.3 months	162	200 MEF	1996-1998	Helsinki	HPeV real time RT-PCR	IV
Respiratory infection patients	0-6 months	170	198 NPA	2001-2004	Tampere	HPeV real time RT-PCR	IV
Hospitalized children with unknown infection	0-13 months	85	79 CSF, 50 serum, 5 stool	2011-2012	Helsinki	HPeV real time RT-PCR	III, IV
NE-patients	13-90y	37	37 serum	2008	Around Finland	LV IFA, HPEV microneutralization	V
Rodents	-	9 and 50	9 serum and 50 blood	2010, 2008	Northern Italy and Konnevesi, Finland	LV IFA	V

AOM, acute otitis media; CSF, cerebrospinal fluid; DIPP, diabetes prediction and prevention study; HPeV, human parechovirus; IFA, immunofluorescent assay; LV, Ljungan virus; MEF, middle-ear fluid; NE, nephropatia epidemica; NPA, nasopharyngeal aspirate

3.1.3 Sera from Finnish adults and from a Dutch population (II)

Serum samples were collected from 72 medical students from the University of Tampere Medical School to represent a Finnish adult population in comparison to child populations. For comparison with the Finnish subjects, 114 serum samples

were obtained from three groups of Dutch individuals: 1-5-year-old children, women of child-bearing age, and HIV-positive men. Each group provided 37-39 samples. All of the individuals visited the Academic Medical Center in Amsterdam, and their serum samples had been directed to virus diagnostics at the Laboratory of Clinical Virology.

3.1.4 Cerebrospinal fluid, serum and stool samples from hospitalised children (III, IV)

Altogether, 79 CSF, 50 serum and 5 faecal samples from 1- to 60-week-old children with a request for microbiological analysis and with no finding of a causative agent, except for EV, were retrospectively collected for HPeV analysis. The children visited hospital, mainly the Helsinki University Hospital, during the period from October 2011 – December 2012.

These samples included CSF, serum and faecal samples from two neonates, which were 4 and 8 weeks old, when hospitalised in autumn 2012; these neonates were described in greater detail in Study III. Originally, the samples were sent to HUSLAB for microbiological analysis, and suspicion of sepsis without bacterial findings directed the samples for EV detection.

3.1.5 Middle ear fluid and nasopharyngeal aspirate specimens (IV)

Middle ear fluid (MEF) samples and nucleic acids, which were extracted from nasopharyngeal aspirate (NPA) specimens, were obtained for HPeV analysis from collaborating groups. The MEF samples were collected from 162 children (2-25 months old) with AOM that originally participated in the Finnish Otitis Media Vaccine Trial during the study period from 1996-1998 [Nokso-Koivisto et al., 2004]. A set of

200 samples, which were previously analysed for a variety of infectious bacterial and viral agents, were included in this study.

Total nucleic acids, which were extracted from 198 NPA samples, were obtained for this study. The NPA samples were obtained from 162 children who were less than 6 months old who participated in a respiratory infection study from November 2001 to May 2002 and from October 2002 to April 2004 [Nuolivirta et al., 2010]. The children had been previously acquainted with hospital care due to bronchiolitis. These samples had been previously analysed for other bacterial and viral human pathogens [Helminen et al., 2008; Nuolivirta et al., 2010], and RSV, RV, influenza-, metapneumo- or adenovirus had been detected in 83% of the samples.

3.1.6 Blood specimens from humans and rodents for LV studies (V)

Sera were sampled from 37 patients with suspected nephropathia epidemica (NE) in 2008 from nine health care districts in Finland. Another four serum samples were acquired from previously HPeV-positive individuals, including a sample from a rodent researcher with frequent rodent contacts. The latter sample was tested and used as the positive control for LV antibody tests.

Serum samples from nine rodents trapped in Northern Italy in 2010 and with previous LV RNA detection in liver samples [Hauffe et al., 2010] were obtained for analysis. Additionally, whole blood samples from 50 bank voles trapped in Konnevesi, Central Finland in 2008 for Puumala hantavirus studies [Razzauti et al., 2013] were analysed.

3.1.7 Parechovirus antisera (V)

LV anti-serum produced in rabbit against VP1 [Tolf et al., 2008] and anti-serum against HPeV1 and HPeV2 produced in horse (VR-1063AS/HO and VR-1064AS/HO; LGC Standards, ATCC, Teddington, United Kingdom) were used as controls for testing LV IFA.

3.1.8 Viruses (II, V)

Virus strains representing HPeV genotypes 1-6 and LV genotypes 1 and 2 were used in this study. Strains were chosen according to their ability to induce a cytopathic effect (CPE). A list of virus strains is presented in Table 5. The strains of HPeV1, 2 and 4-6 were originally from Dutch clinical samples, whereas the HPeV3 strain was isolated in this study (I). The HPeV1 Harris strain was used as a control. The LV strains used in this study included cell culture condition-adapted LV1 and 2 isolates.

Table 5 Virus strains used in this study.

Genotype	Strain	Reference
HPeV1	152212	Benschop et al., 2006b
HPeV1	Harris	Hyypia et al., 1992
HPeV2	751312	van der Sanden et al., 2008
HPeV3	FI0688	Study I
HPeV4	K251176-02	Benschop et al., 2006a
HPeV5	20552322	Benschop et al., 2006b
HPeV6	20751393	Benschop et al., 2008b
LV1	87-012G	Johansson et al., 2004
LV2	145SLG	Tolf et al., 2008

3.1.9 Cell lines

Viruses were cultured in American Type Culture Collection (ATCC) cell lines originating from different human and monkey tissues, as well as in GMK (Green monkey kidney) cells. A549 (human alveolar epithelial adenocarcinoma), Caco-2 (human colon carcinoma), GMK, HeLa (human cervical epithelial carcinoma), HT29 (human colon adenocarcinoma), LLC-MK2 (rhesus macaque kidney), Vero (grivet kidney) and Vero E6 (grivet kidney) cells were maintained in minimum essential

medium (MEM), Dulbecco's modified Eagle's medium (DMEM) or F-12 nutrient medium with 10% heat-inactivated foetal bovine serum (FBS), 100 IU/l penicillin and 10 µg/ml streptomycin at +37°C and 5% CO_2. For virus propagation, the amount of FBS was decreased to 1-5%, depending on the cell line.

3.1.10 Reference sequence material from databases

The phylogenetic analysis in this study was conducted in comparison to reference sequences and to other published sequences available in the GenBank database (www.ncbi.nlm.nih.gov/genbank) of the National Center for Biotechnology Information (NCBI). In addition to the reference strains of HPeV genotypes 1-16, 3 HPeV1, 3 HPeV3, 42 HPeV4 and two HPEV6 isolate sequences were included in the phylogenetic relation analysis.

3.2 Methods

3.2.1 Virus isolation in cell cultures (I, III and IV)

Parechovirus strain cultivation was tested in different cell lines, particularly for setting up the microneutralisation assay. The best cell lines were the ATCC-cell lines HT-29, Vero and Vero E6. HPeV1, 2 and 4-6 infected HT-29 cells and induced a distinguishable CPE. Vero and Vero E6 cells were better suited for cultivating HPeV3 because no CPE occurred in HT-29 cells. The LV strains were previously adapted to the cell culture conditions and were cultivated in GMK and Vero cells.

The cultivation and isolation of HPeV was attempted from faecal (I, III and IV), CSF (IV) and serum samples (III, IV). The culture medium was discharged from the cells, grown to 50-70% confluence in a 25-cm^2 culture flask or in a 5.5-cm^2 culture tube with a flat side before the addition of 50-150 µl serum, CSF or 10% (w/v) homogenised faecal suspension sample. After 1 h of incubation, fresh DMEM supplemented with 2% FBS and antibiotics was added to cells inoculated with serum

or CSF sample. The same incubation time was used for faecal suspensions; however, these samples were discharged before the addition of fresh MEM or DMEM supplemented with 2% FBS, penicillin, streptomycin, gentamicin, and amphotericin B to the cells. The time of induction and the level of induced CPE varied between isolates. CPE was primarily detectable after 4 days, although the incubation was continued for up to 4 weeks for some isolates. Then, the virus was freed into the supernatant with three rounds of freeze-thaw cycles. The presence of the virus in the supernatant was further controlled with RT-PCR or with real-time RT-PCR before storing at -70°C for further studies.

3.2.2 RNA extraction (I, III and IV)

Viral RNA was extracted from various materials for RT-PCR-based detection analyses. Extraction from CSF, faecal, serum, MEF and cell culture samples was performed using a QIAamp Viral RNA kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Total nucleic acids from NPA specimens were extracted using a High Pure Template Preparation kit (Roche Applied Science, Indianapolis, IN, USA). Most RNA samples were stored at -70°C, and total nucleic acid samples were stored at -20°C until analysis.

3.2.3 RT-PCR coupled with liquid hybridisation (I)

HPeV RNA detection from samples was based on PCR targeted to a conserved sequence in the 5'-end of HPeV genome. The RT-PCR coupled with hybridisation was conducted as described previously [Tauriainen et al., 2007; Tauriainen et al., 2008]. This method was used to detect viral RNA before the introduction of real-time RT-PCR methods. This method included a reverse transcription step using an HPeV-specific primer (Par30) and a PCR step with a biotin-labelled primer (Par28-bio,

Table 6). In this method, the amplified PCR product was fixed to the bottom of the plate by biotin-streptavidin binding, and the PCR amplicon was detected by hybridisation to a europium-labelled probe.

Table 6 Oligonucleotide primers and probe used in HPeV detection and genotyping. Genome position according to the HPeV1 Harris strain (GenBank accession no. S45208).

Name	Sequence (5' to 3')	Genome position	Reference
Par28-bio	biotin-AGCCATCCTCTAGTAAGTTTG	313-333	Modified from Oberste et al. [1999]
Par30	GGTACCTTCTGGGCATCCTTC	577-556	Oberste et al. [1999]
Par31	CTGGGGCCAAAAGCCA	441-457	Benschop et al. [2008a]
HPeV probe	6'FAM-AAACACTAGTTGTA(A/T/C)GGCCC-MGB-NFQ	535-554	Modified from Benschop et al. [2008a]
HPeV_VP1f	ATTC(A/G)TGGGG(C/T)TC(A/C)CA(A/G)ATGG	2337-2357	Study I
HPeV_VP1rev	AATATCCTTAGAAT(A/G/T)GT(C/T)TCACA(A/G)TT	3328-3302	Study I

FAM, 6-carboxylfluorescein, MGB, minor groove binder; NFQ, non-fluorescent quencher

3.2.4 One- and two-step real-time RT-PCR (III and IV)

HPeV RNA detection included real-time PCR protocols with one step and two steps, which were both adapted and modified from Benschop et al. [2008a]. The cDNA synthesis for the two-step reaction was performed in a 40 µl reaction containing 10 µl of RNA template, 8 µl of reaction buffer, 50 pmol of HPeV-specific primer (Par 30, Table 6), 20 nmol of dNTP, 4 units of Recombinant RNasin© RNase inhibitor and 20 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA).

Additionally, 0.5% bovine serum albumin (BSA) was added to faecal suspension samples to minimise RT and PCR reaction inhibition. The reaction mixture was incubated for 1 h at 37°C to complete the reaction.

The PCR step was performed using a Maxima qPCR master mix kit (Thermo Scientific, Rockford, IL, USA) in a 25 µl reaction, which contained 5 µl of cDNA product, 300 nM of primers (Par30 and Par31, Table 6), and 200 nM of probe (HPeV-

NedA). The Taqman® probe included the reporter label 6-carboxylfluorescein at the 5'-end and a minor groove binder and non-fluorescent quencher at the 3'-end. An ABI 5' Prism 7900HT System (Applied Biosystems, Foster City, CA, USA) was used to perform the assay with following parameters: 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C.

One-step RT-PCR was set up to increase the sensitivity and speed of the HPeV detection assay. The same set of primers and probe as in the two-step system was utilised in this reaction, which was performed using the Superscript® III Platinum® One-step qRT-PCR System (Invitrogen, Carlsbad, CA, USA). The total reaction volume of 25 µl included 12.5 µl of reaction mix, 7 µl of RNA template, 400 nM of each primer and 200 nM of the fluorogenic probe. The RT-PCR reaction, whose conditions were 15 min at 50°C and 2 min at 95°C, followed by 45 times of a two-step cycle of 15 s at 95°C and 50 s at 60°C, was performed using a Stratagene Mx3005P qPCR System (Agilent Technologies, Santa Clara, CA, USA).

3.2.5 HPeV typing PCR (I, III and IV)

An RT-PCR targeted to the almost complete VP1-sequence area of the HPeV genome was developed for genotyping HPeV-positive findings as part of this study (I). The typing was performed using viral RNA directly extracted from the target sample or from the cell culture suspension after virus propagation from the sample. cDNA synthesis was performed as described in 3.2.4, except with a different specific primer (HPEV_VP1rev, Table 6).

The PCR reaction was performed using Biotools Taq polymerase (Biotools, Madrid, ESP) in a final volume of 50 µl, which contained 5 µl of cDNA template, 10 µl of reaction buffer, 200 nM of each primer (HPeV_VP1f and –rev, Table 6), 200 µM of dNTP and 3 units of Taq DNA polymerase. The VP1 PCR conditions were as follows: 94°C for 2 min, followed by 36 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 2 min, with a final extension at 72°C for 10 min.

The positive PCR fragments observed by agarose gel electrophoresis were directed to sequencing using the same primers used in the typing RT-PCR. Sequencing reactions were performed by MACROGEN® (Seoul, Korea) or by Helsinki University sequencing service. In the latter, the PCR products were purified using a Qiaquick gel extraction kit or a Qiaquick PCR purification kit (Qiagen Inc.) before the sequencing reaction.

3.2.6 Sequence analysis and phylogeny (I and IV)

The data from genotyping the VP1 sequences was initially compared with reference sequences using BLAST [Altschul et al., 1990] and aligned using the CLUSTALW tool [Thompson et al., 1994]. In Study I, the estimation of evolutionary relations was conducted using the neighbour-joining method with the Kimura two-parameter model [Kimura, 1980] and using the DNAdist program contained in the PHYLIP software package [Felsenstein, 1993]. In Study IV, the neighbour-joining method was used with Tamura-Nei algorithm [Tamura and Nei, 1993] using MEGA 6.6 software [Tamura et al., 2013]. The evolutionary distances were estimated with 1000 bootstrap pseudoreplicates [Hillis and Bull, 1993].

3.2.7 Statistical analysis (I, II)

Differences in HPeV frequencies between boys and girls and different locations, as well as the significance in the differences in the level of neutralising antibodies between groups were analysed using the X^2-test in IBM SPSS statistics 19 software. P-values of <0.05 were considered statistically significant.

3.2.8 HPeV microneutralisation assay (II, V)

A microneutralisation assay for HPeV types 1 to 6 was developed and set up as part of this study (II) using virus titres of 50 $TCID_{50}$ per 2.5 µl. The assay for detection of neutralising antibodies against HPeV 1, 2, 4, 5 and 6 used HT29 cells, whereas Vero E6 cells were used for HPeV3 antibodies. The serum samples were diluted fourfold (1:8–1:4096) in Hank's balanced salt solution (including $CaCl_2$ and $MgCl_2$). A 2.5 µl volume of serum dilution was mixed with 2.5 µl of virus and incubated for 1 h at 37°C, followed by overnight incubation at room temperature. Then, the mixture was inoculated to a confluent monolayer of cells on microtitre plates. DMEM growth medium supplemented with 2% FBS was added to each well, and the cells were grown for 5-7 days before staining with crystal violet. The HPeV types grown on HT29 cells caused a CPE, which resulted in the cells detaching from the bottom of the well unless neutralising antibodies were present. In contrast, HPeV3 infection turned the cells dark and round (CPE) without detaching, and this result was observed under a light microscope. Serum dilution was considered positive when 50% or more of the infection was prevented, and the lowest dilution considered positive was 1:16.

3.2.9 LV microneutralisation (V)

The LV microneutralisation assay was performed in comparison to the immunofluorescence assay (IFA) using LV strain 145SLG in a titre of 60–100 $TCID_{50}$ and Vero cells. Serum samples were inactivated performed before the assay using a heat treatment for 30 min at 56°C. Fourfold serum dilutions ranging from 1/8 to 1/512 were prepared using Hank's balanced salt solution (HBSS; University of Helsinki). The LV was mixed with serum dilutions and incubated for 1 h at 37°C before adding to Vero cells. The formation of infection foci on the monolayer of cells, which were grown on 96-well plates in media consisting of MEM supplemented with 2% FBS and 1X GLUTPEST (Invitrogen), was monitored daily for 5 days using a light microscope. Then, the cells were stained with crystal violet to observe the plaques. The number of plaques in the presence of serial serum dilutions was compared with that in the virus

control to calculate the neutralisation effect. A reduction in the number of infected cells of at least 80% in comparison to LV control wells was considered positive.

3.2.10 LV indirect fluorescence assay (V)

LV IFA was set up with LV strains 87-012G and 145SLG (Table 5) and with Vero cells. The IFA protocol was modified from Kallio-Kokko et al. [2001]. Briefly, LV-infected Vero cells with weak signs of CPE were collected and washed 5 times with PBS before mixing at a ratio of 1:1 with non-infected cells. The mixture was further diluted with PBS before adding to 10-well diagnostic slides (Paul Marienfeld GmbH and Co. KG, Lauda-Königshofen, Germany). The cells were dried in the wells overnight at room temperature before fixing for 7 min with ice-cold acetone (Sigma-Aldrich Finland, Finland).

Dilutions of serum samples (1:20) and rodent whole blood samples (1:10) in PBS were added onto diagnostic slides before incubation for 30 min at 37°C, followed by washing three times with PBS and once with water for 5 min before drying. For slides with human serum samples, fluorescein (FITC)-AffiniPure F(ab')$_2$ fragment goat anti-human IgG (H+L) (Jackson Immuno Research Laboratories, West Grove, PA) diluted 1:100 was added next and incubated for 30 min at 37°C.

Similar incubations were performed, with 1:100 dilution of FITC-AffiniPure goat anti-horse IgG (H + L; Jackson ImmunoResearch Laboratories) for HPeV1 and HPeV2 antisera produced in horse and with 1:30 dilution of polyclonal rabbit anti-mouse immunoglobulins labelled with FITC (all mouse IgG subclasses, mouse IgM and mouse IgA; Dako Finland Oy, Helsinki, Finland) and of polyclonal swine anti-rabbit immunoglobulins labelled with FITC (immunoglobulins of all classes, Dako Finland Oy) for rodent samples and antisera produced in rabbit. Then, the slides were washed as described previously and dried before adding cover slips with mounting

medium. The results were detectable using a fluorescence microscope (Leica Microsystems, Espoo, Finland) with an FITC-filter.

3.2.11 Electron microscopy (III)

A cell culture suspension sample of HPeV4 strain FI121236-infected cells was fixed onto copper grids, stained by negative staining with 2% KPTA (tungstophosphoric acid) and examined using a JEOL JEM 1400 electron microscope.

4 Results and Discussion

4.1 HPeV prevalence and seasonality (I)

In the majority of previously conducted studies, HPeV has been screened for and detected in clinical samples. Therefore, knowledge regarding their presence and circulation in background populations has remained scarce. In this study, we detected HPeV in stool samples from 78 of 200 (39%) healthy children. By 12 months old, 22% of the sampled children had at least one HPeV-positive sample, and by 22 months, this percentage increased to 48%. After 2 years old, the rate of HPeV detections decreased. Fifty percent of the findings had two or more consecutive HPeV-positive samples with shedding up to 93 days. Eight children had two distinct HPeV episodes. Detection rates were similar between Turku and Tampere, as well as between boys and girls. HPeV was detectable from samples collected between 1996 and 2006; however, this detection distributed unevenly due to the variation in the sample number for different years (Figure 3). HPeV was absent in the small number of samples collected in 2007. During most years, a distinct peak in HPeV detections was observable from October to November. The HPeV genotype was identified for 105 (73%) of 144 positive samples. The primary type detected was HPeV1, which was typed 98 times, whereas HPeV6 was identified in four samples, and HPeV3 was identified in three samples.

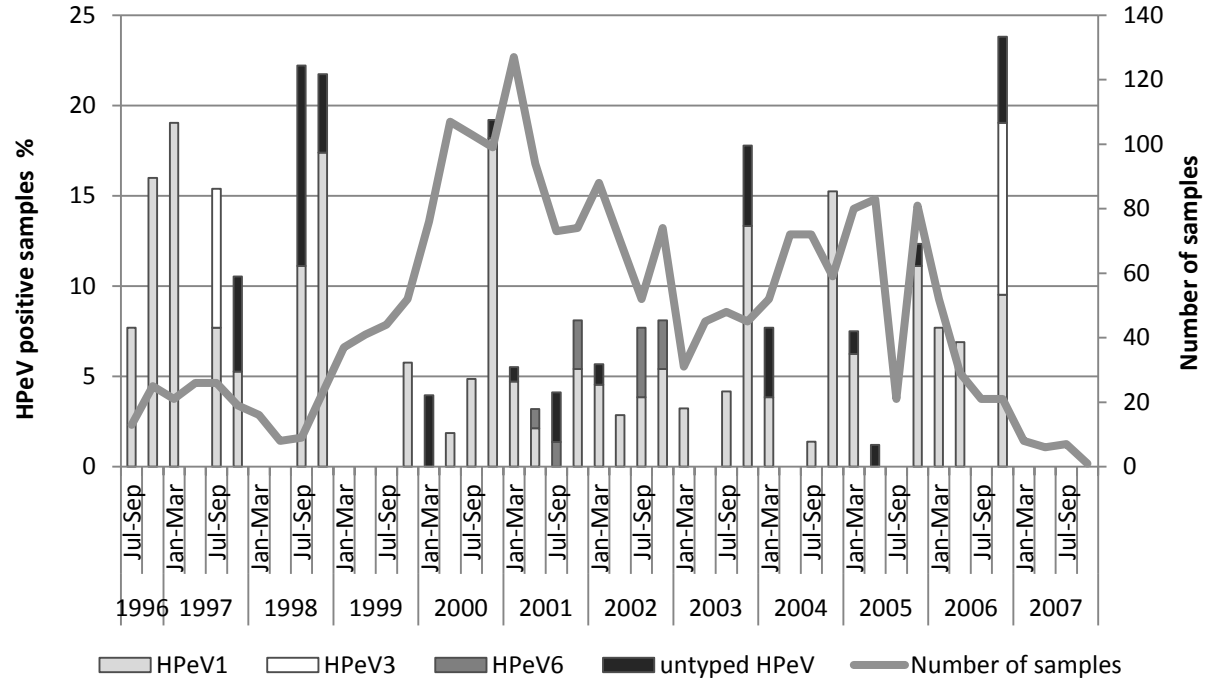

Figure 3 Timing and percentage of HPeV-positive stool samples during the study period from 1996-2007. The results are shown in three-month groupings (January-March, April-June, July-September and October-December).

These results demonstrate that HPeVs commonly circulate among healthy Finnish children. The actual number of HPeV-positive children is likely to be even higher because continuous, monthly sampling series of 18 or more samples, which were collected until two years old, were available from only 36 (18%) children. The majority of sample series comprised a lower number of samples; thus, the question of whether HPeV remained undetected was left unanswered. Additionally, the presence of HPeV was not studied in all of the children before three months old. A Norwegian study with a similar setting and with a more consistent sample series demonstrated that 86% of 102 children had their first HPeV infection before reaching two years old [Tapia et al., 2008]. The long-lasting shedding of HPeV1 suggests that this virus is detectable using a sampling frequency of collecting one sample each month. Long-lasting shedding may also explain the common occurrence of HPeV1 because this characteristic allows more opportunities for transmission to new hosts.

The children recruited to the DIPP study were healthy and, therefore, were good representatives of the background population. However, the children were selected for the follow-up study according to certain criteria, which limit the generalisation of the results. The collection of monthly samples from healthy individuals at an identical scale to that used in the DIPP study is highly challenging; therefore, this sample series is unique for analysing the circulation of viral agents in the Finnish population.

The HPeV genotypes circulating among healthy Finnish children include at least HPeV1, 3 and 6. Consistent with our results, HPeV1 has been widely reported as the most commonly detected HPeV genotype and is followed in frequency by genotypes 3, 4 and 6 [Benschop et al., 2008b; Boros et al., 2010; Pham et al., 2011; Tapia et al., 2008; Zhang et al., 2011]. Except for a single finding of HPeV14 in the Netherlands [Benschop et al., 2008b], the genotypes commonly circulating in Europe appear to be HPeV 1 to 6. In rare cases, the presence of HPeV2 and 5 has also been reported. Thus, the genotype distribution pattern in Finland is similar to that reported elsewhere in Europe. No HPeV4 was detected in this sample series, which is consistent with data analysed in Norway [Tapia et al., 2008] and in Scotland [Harvala et al., 2008]. In contrast to this observation, HPeV4 was detectable later in Finland during a series of neonatal infection cases (IV).

HPeV circulation was observable throughout the year during the study period from 1996 to 2006, with a seasonal peak from October to November during most years. Studies from many other countries have described similar seasonal pattern [Benschop et al., 2006b; Tapia et al., 2008; Zhang et al., 2011]. In contrast, in China in 2009, a temporal change in the HPeV prevalence from autumn to summer was reported [Guo et al., 2013]. Because we primarily detected HPeV1, we could base our seasonality analysis solely on this genotype. The detected numbers of HPeV3 and 6 were too low to consider for a more detailed analysis. However, if these numbers are considered, then the few detections of HPeV3 do not support the biennial cycle with a peak during summer months [Benschop et al., 2008b; Harvala et al., 2011; van der Sanden et al., 2008], which has been suggested for HPeV3.

The results from our study are consistent with a later study conducted by Simonen-Tikka and colleagues (2013), which similarly describes a long circulation and shedding period of HPeVs, in addition to their seasonal prevalence in autumn in Finland.

Serum samples were tested for neutralising antibodies (nABs) against HPeV types 1 to 6 to examine whether a monthly sampling frequency represents a sufficiently short interval for HPeV detection. Samples of seven children with an HPeV-negative consecutive series of stool samples between the ages of 3 and 24 months were collected at 24 months old. All of the seven samples were negative for nABs against HPeV1, 3 and 4. However, four children had nABs against HPeV2, whereas one of the children also had nABs against HPeV5 and 6.

Our neutralisation data supports the observation that the monthly collection of stool samples represents a sufficient frequency for HPeV1 detection. In contrast, the presence of nABs against HPeV2 in four of seven serum samples is intriguing because HPeV2 was not detectable in the stool samples. This discrepancy raises a question regarding the validity of these findings. Interestingly, similar divergences were also observed in the analysis of nABs in Study II.

4.2 Seroprevalence of HPeV (II)

NABs against HPeV types 1 to 6 were common in Finnish and Dutch populations. In adults, the seroprevalence was high for HPeV1 and HPeV2 (86-92%), moderate for HPeV4-6 (35-75%) and low for HPeV3 (10-13%). Due to differences in the sample selection process, the age groups of children from the two countries were incomparable. We conducted a comparison between Finnish and Dutch adult populations, although the selection of individuals in the populations differed and was only roughly comparable. The seroprevalence for HPeV1 to 4 was similar in Finnish and Dutch adults, whereas nABs against HPeV5 (35% vs. 75%; p= <0.001) and 6 (57% vs. 74%; p= 0.04) were significantly more common in Dutch adults.

The seroprevalence increased with the age of the study populations (Figure 4). Interestingly, the highest seropositivity level for most HPeV genotypes tested, i.e., the level attained in adulthood, was already reached in children by 5 years old. The seroprevalence of HPeV3 was noticeably higher in adults compared with that of 5-year-olds.

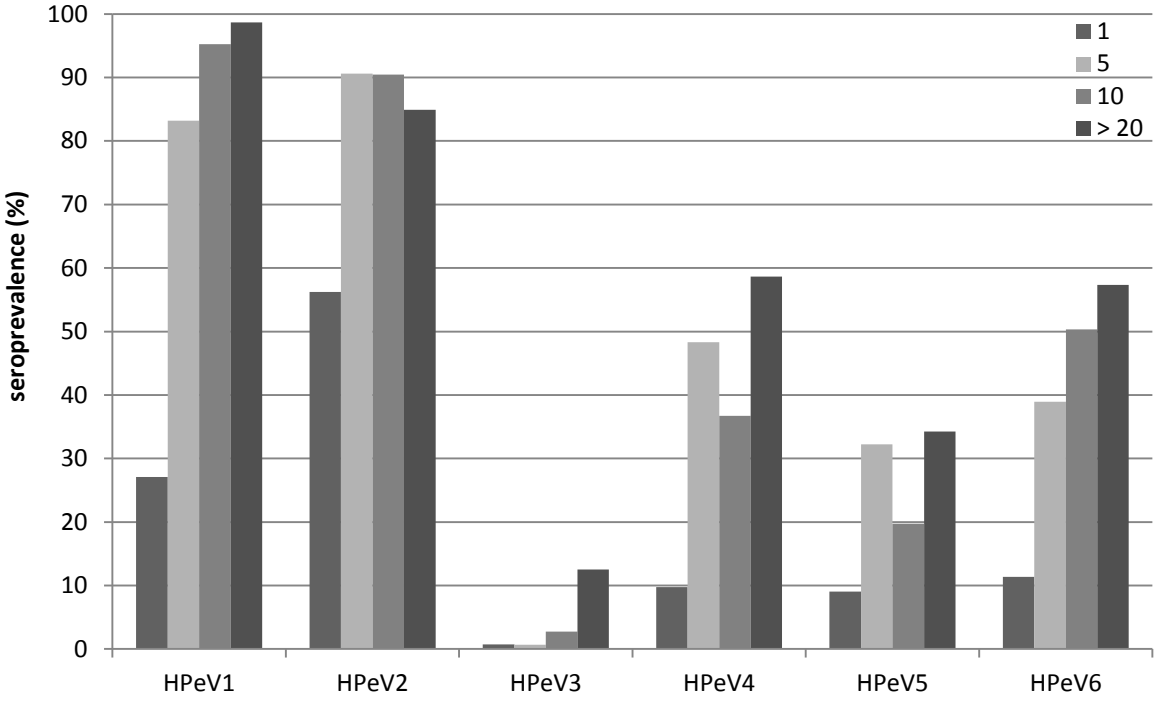

Figure 4 Seroprevalence for HPeV1-6 in Finnish age groups of 1-, 5-, and 10-year-old children and in adults (>20 years old).

The high seroprevalence detected for HPeVs in this study suggests that HPeVs are even more common than previously assumed. Although the serologically abundant nature of HPeV1 has been previously discovered [Joki-Korpela and Hyypia, 1998; Tauriainen et al., 2007], knowledge regarding the seroprevalence of other HPeV genotypes has been scarce thus far. Only one study, namely, the study conducted by Ito and co-workers (2004), handled similar serological data on HPeV3. To our knowledge, no previous reports regarding the seroprevalence of HPeV4 to HPeV6 have been published. Most of the other epidemiological data available are based on stool sample analyses of hospitalised patients.

Our results further reveal the presence of HPeV4 and HPeV5 in the Finnish population. This finding was particularly interesting because the two genotypes had never been detectable in Finland before 2012. Overall, the serological data were primarily consistent with prior available results from stool sample analyses of hospitalised patients in European countries, with the exception of HPeV3 being less prevalent and HPeV2 being far more prevalent than observed previously.

The selection criteria for the different populations in this study limit the generalisation of the results and the comparison of the subgroups from Finland and the Netherlands. However, these results do provide the first insights into the seroprevalence of multiple HPeV types in different populations. Another limitation of this study was the uncertainty of potential cross-reaction in the detection of type-specific nABs. Although no clear pattern of this effect was observable, the possibility of affecting the results on some level cannot be ignored.

High seroprevalence for HPeV2 is intriguing because this genotype has rarely been detected and isolated from human samples. Cross-reactivity between HPeV2 and other genotypes represents one explanation; thus, this finding would be an irreverent by-product of neutralisation testing. However, this possibility appears unlikely because an extensive number of children (60 children of 441 in Finnish data) were positive only for HPeV2. Additionally, we detected no indication of a sample being HPeV2 seropositive each time antibodies against any other genotype were present. Moreover, only extremely weak cross-reactivity was reported against HPeV2 by antisera of other HPeV types, whereas the HPeV2 antiserum did not neutralise other HPeV types, indicating specificity at least compared with HPeV types 1 to 6 [Westerhuis et al., 2013]. Although cross-reactivity among different HPeV types can be excluded, the detected HPeV2 antibodies cannot be conclusively verified as not being antibodies against some other virus with antigenic epitopes resembling those epitopes of HPeV2. In contrast, the discrepancy between rare isolation and high seroprevalence might either be attributed to a shorter shedding period, demanding the requirement for sampling interval shorter than per month, or to a low level of replication, leaving the viral count below detection limits.

Because HPeV2 was not detectable in stool samples (I), one might assume a different primary replication site than the intestine. However, we detected no HPeV2 RNA in NPA, MEF, CSF or serum samples (IV) nor has HPeV2 RNA been found in other studies testing various sample types [Chen et al., 2009; Harvala et al., 2014; Nielsen et al., 2013]; thus, this possibility can be excluded.

We detected only 13% and 10% seroprevalence for HPeV3 in adults in Finland and in the Netherlands, respectively, which is significantly lower than that reported previously in Japan (87%) [Ito et al., 2004]. The nAB level was even lower in children. The retrospective analysis of neutralising antibodies from three children with HPeV3 detection in the stool demonstrated that the presence of HPeV3 did not initiate the production of any nABs in one of three cases. NABs were present in the remaining two cases; however, in one of the cases, the level of nABs decreased below the detection limit within a few months. Unfortunately, we only had three stool-positive children and, therefore, were unable to further confirm this effect. However, the absence of nABs against HPeV3 has also been reported previously [Mizuta et al., 2012; Westerhuis et al., 2012]. Additionally, the antibodies raised against HPeV3 did not have a neutralising effect [Westerhuis et al., 2013]. Therefore, HPeV3 has likely employed a strategy of avoiding its own neutralisation.

The relatively recent emergence of HPeV3 [Calvert et al., 2010] may explain its diverging pathogenicity from that of other HPeV types. Its recent spread would account for reduced adult exposure and, thus, reduced production of protective maternal antibodies, making neonates more susceptible to HPeV3 infections [Harvala et al., 2010]. This theory is supported by a report describing HPeV3 infections detected simultaneously in a mother and in her young children [Al Maamari et al., 2009]. The partially immature immune system might also contribute to the higher risk of HPeV3 infection in neonates [Wildenbeest et al., 2010]. Our results indicate another potential explanation for the stronger pathogenicity of HPeV3, namely, the observation that this virus avoids its neutralisation by antibodies.

4.3 Description of clinical and virological findings in two neonatal HPeV4 infection patients (III)

In October 2012, two male neonates, who were 8 (case 1) and 4 (case 2) weeks old, were hospitalised due to suspected infection. At six weeks old, case 1 had already been hospitalised with pneumonia and treated with G-penicillin. However, the causative agent that induced the pneumonia remained unknown. Following the treatment for pneumonia, the case 1 neonate was discharged in good clinical condition from the hospital until he was readmitted two weeks later due to suspected bacterial sepsis. The patient presented with high fever, tachycardia (220-230/min), and clear irritability. The blood parameters were within the normal range (detailed in III). To rule out recurring pneumonia, the chest was X-rayed, and no sign of inflammation was detected. The patient remained hospitalised for seven days and was treated for the entire period with G-penicillin and for three days with acyclovir after initial treatment with intravenous cefuroxime.

The case 2 neonate, who was only 4 weeks old, was hospitalised, presenting with fever and leukopenia. In addition to fever, the patient showed signs of skin marbling but no other abnormalities when examined physically. The blood parameters of the patient were normal, with a slightly decreased level of leukocytes (3.8×10^9/l). The chest X-ray was normal, whereas a slight elevation of protein content was obviousble in CSF samples. A macular rash, with round lesions two to three millimetres in diameter, became apparent on the patient's skin after three days of hospitalisation. After hospitalisation, the initial treatment of case 2 with G-penicillin was changed to a course of five days ampicillin, in addition to cefotaxime and three days of acyclovir.

After extensive microbiological testing for causative agents of viral or bacterial nature at the diagnostic laboratory of HUSLAB (Finland), the only progress was the detection of an EV-like CPE in viral cultures from faecal samples of both neonates. However, specific EV screening remained unsuccessful. Therefore, the patients' samples were redirected to our department for HPeV detection, which was a method not yet included in HUSLAB routine diagnostics.

HPeV4 RNA was detectable from both neonates in viral cultures from faecal and serum samples but was absent in CSF samples, which were also negative for all other tested pathogens. Picornavirus- sized virus particles were detectable by EM (Figure 5), further confirming the presence of an HPeV. During hospitalisation, the cell counts of case 2 normalised, and both children were discharged in clinically good condition after six to seven days of hospitalisation.

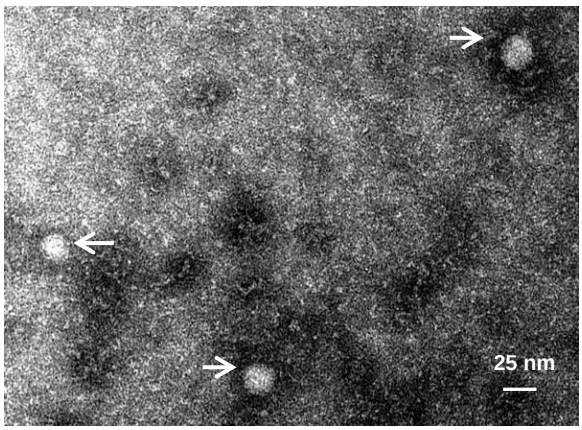

Figure 5 HPeV4 virus particles, marked with arrows, in a virus culture sample on an EM-image.

The two cases described in the present study demonstrate that HPeV4 circulates in Finland and is detectable in the serum and stool from neonates with severe infections. Previously, HPeV4 has been associated with mild diseases, whereas these neonates had been hospitalised with sepsis-like symptoms, similar to those symptoms observed in children with neonatal HPeV3 infection. One of the neonates also developed dermatological changes, which have not been previously reported in HPeV4 cases but which are typical for HPeV3 infection [Levorson et al., 2009; Shoji et al., 2013].

4.4 HPeV infections in young children with severe diseases (III, IV)

The aforementioned cases in neonates represent not only the first finding of HPeV4 infections in Finland but also some of the first HPeV-related infections of a higher severity in Finland, raising our interest in this particular genotype and in other genotypes linked to more severe disease manifestations. To obtain a better understanding of their epidemiological character in young children with severe clinical presentations in Finland, we designed a retrospective screening approach. Samples from 85 children with suspected CNS infection, which were collected during the period from October 2011 to December 2012, were screened for HPeV. Seven (8%) children, including the two cases described in Study III, were HPeV-positive in CSF, serum or faecal samples. Five of the HPeV detections were from samples collected from September to October 2012, and four of these samples were successfully typed as HPeV4, whereas one case remained untyped. These children were two to eight weeks old. Additionally, a six-week-old child was detected to have had an HPeV3 infection in October 2011, and a 13-month-old child an untyped HPeV infection in May 2012. During routine diagnostics (HUSLAB, Finland), the children participating the study had been tested using real-time RT-PCR for EV, indicating that six of these children were EV-positive. No child had a simultaneous infection of both HPeV and EV.

All of the HPeV-positive children were admitted to hospital care and had tested negative for other infectious agents. The symptoms (Table 7) of the patients varied; however, each patient had a fever. The patient infected with HPeV in May 2012 was older (56 weeks old) than the other patients, who were two to eight weeks old. This child had presented with unusual neuroimaging features, fitting the criteria of acute disseminated encephalomyelitis (ADEM). This child had further presented with slightly elevated platelet counts, with an increased level of CSF protein in addition to leucocytosis in serum. Four of the other children had had suspected sepsis, with fever, rash, poor feeding and irritability.

The laboratory results indicated leukopenia for three children, whereas CSF protein counts were slightly elevated in three patients, including the ADEM patient. Unfortunately, the CSF laboratory results were available for only some of the HPeV-positive cases (laboratory findings for each child are presented in Study IV, Table 3).

Table 7 Clinical features of HPeV-infected young children with suspected CNS-infection.

Feature	Number of cases
prolonged fever	7/7 (100%)
suspected sepsis	4/7 (57%)
leucopoenia	3/7 (43%)
tachycardia	1/7 (14%)
rash	1/7 (14%)
acute disseminated encephalomyelitis (ADEM)	1/7 (14%)

These seven cases are among the first severe HPeV infections detected in Finland. The only HPeV infection case with a more severe outcome before these findings represents a single case of HPeV1-induced encephalitis in 1989 [Koskiniemi et al., 1989]. Our retrospective screening approach revealed that all except one of the HPeV-positive patients had HPeV4 in autumn 2012. The single HPeV infection case without a known genotype remained untyped because no positive sample material from this child was left for genotyping. All of these patients were positive for HPeV in serum, whereas two of these patients were also positive in CSF, and two others were positive in faecal samples. Because HPeV4 has never been detected before in Finland, its role, as well as that of other HPeVs, in clinical infections of Finnish children was previously unknown. Thus, no apparent reason could be given for HPeV detection as part of Finnish routine diagnostics before this study. Past unsolved infectious cases fitting patient and clinical profiles may represent undetected HPeV infections. No knowledge regarding the potential role of HPeV4 infections in 2012 outside the Uusimaa district in Finland exist because our analysed sample pool was limited to this area.

The cases of HPeV4 observed in this study may have been part of a local outbreak. We only detected HPeV from children with suspected CNS infection because our focus was on patients with more severe symptoms. Thus, whether clinically milder infections can also be linked to HPeV4 in Finland remains to be investigated. However, outbreaks of other picornaviruses have been reported previously in Finland [Savolainen-Kopra et al., 2011]. HPeV3 outbreaks have even been described in other countries [Nordbø et al., 2013; Sharp et al., 2012b].

The serological data (II) demonstrate that HPeV4 has already been circulating in Finland for some time. Thus, an alternative explanation for these neonatal HPeV4 cases may be the lack of specific, protecting maternal antibodies, which would predispose a child to HPeV infection. Eight percent of 1-year-old children in Study II had neutralising antibodies against HPeV4, suggesting that thousands of Finnish children may be exposed to HPeV4 before that age. Furthermore, of the Finnish adult population and Dutch mothers, approximately 60% had HPeV4 antibodies, leaving a large part of children unprotected by maternal antibodies during their first months of life.

Studies regarding neonatal sepsis during the first month of life revealed that bacterial causes were responsible in only 10 to 15% of tested cases, whereas the rest were assumed to be virally induced, with EVs being the primary viral cause [Byington et al., 2004]. However, the causative agent remained unsolved in many of these cases. Although no HPeV4 has yet been detected in studies of HPeV-associated neonatal diseases, HPeV4 may represent one of the undetected causative agents [Benschop et al., 2006b; Harvala et al., 2011; Walters et al., 2011]. In addition to fever [Benschop et al., 2006a], HPeV4 has previously been associated with respiratory and gastrointestinal symptoms [Pajkrt et al., 2009]. Recently, HPeV4 was detectable in a single case of sepsis-like disease in France [Jeziorsky et al., 2014], a finding similar to the cases detected in this study. Although this recently detected HPeV4 may represent a newly evolved, genetically different HPeV4, we could not observe sufficient genetic differences in the sequenced VP1 region to support this assumption.

One of the HPeV-positive patients of our retrospective study, a patient with suspected sepsis, tested positive for HPeV3 in a faecal sample. The previous detections of HPeV3 in Finland are from healthy children (I). The other HPeV-positive child with a differing infection pattern had an untyped HPeV in May 2012. This patient was slightly older and had ADEM, which has not been previously associated with HPeV-induced infection. Unfortunately, we were not able to obtain other samples or information regarding the underlying diseases from these two patients. Thus, the causative relation of HPeV in relation to the clinical case presentation can only be suspected but not confirmed with certainty.

Neonates with a manifested infection of unknown nature and presenting with CNS-related or sepsis-like disease symptoms are hospitalised and treated with broad-spectrum antimicrobials to prevent any spreading or severe damage caused by the infectious agent(s). Once the infectious agent is identified, then the amount of medication may be reduced and changed to a more targeted treatment. Therefore, determining the cause of a neonatal infection in the shortest possible timeframe is extremely important. At present, however, a large fraction remains without a definite diagnosis. Although no specific treatment is available for EV and HPeV infections, their detection is vital for choosing the right treatment. Based on our findings, HPeV detection methods were added to routine diagnostics in Finland, representing an advance in paediatric care.

4.5 Association of HPeV to acute otitis media and to respiratory infections (IV)

As shown in Study I, the occurrence of HPeV is common in healthy children and, thus, often causes only mild or unapparent infections. The association of HPeV with common diseases of young children has been suggested in many studies [Ghazi et al., 2012; Ito et al., 2010; Khetsuriani et al., 2006; Pajkrt et al., 2009]. We examined the association of HPeV in two highly common disease groups of children: AOM patients and bronchiolitis patients.

Although the prevalence of HPeV was low among AOM cases, HPeV was absent from all of the 198 NPA samples from bronchiolitis patients. In AOM patients, HPeV was detectable in MEF samples in 5 of 200 (2.5%) infection events, of which two were successfully typed as HPeV1. These results indicate no connection between HPeV and these common paediatric diseases.

The most common cause of AOM is considered a co-infection of bacterial and viral pathogens [Monobe et al., 2003; Ruohola et al., 2006]. Furthermore, the bacterial infection in AOM may follow an initial viral respiratory infection [Heikkinen and Chonmaitree, 2003]. Therefore, the association of a virus with AOM is often studied using both MEF and NPA samples. Our lack of data regarding the presence of HPeV in NPA samples of AOM patients limits the conclusion that HPeV has no association with the onset of AOM.

HPeV1 is one of the many potential human pathogens suggested to cause AOM [Tauriainen et al., 2008]. However, the detection rate of other viruses, such as RV, in MEF samples from AOM patients is much higher than that of HPeV [Savolainen-Kopra et al., 2009]. Although we detected only HPeV1 in AOM patients, other studies have shown HPeV4, 5 and 6 in NPA samples from patients with AOM [Pajkrt et al., 2009] but could not deliver proof of causality. The presence of different HPeV types in AOM patients is evident, whereas the association with the disease remains to be confirmed.

The role of HPeV in the onset of respiratory diseases is uncertain because HPeVs have only been detectable in low rates in respiratory infection patients [Harvala et al., 2008; Khetsuriani et al., 2006; Selvarangan et al., 2011]. A recent study has suggested that HPeV is as common in respiratory samples from patients with acute respiratory illness as in samples from healthy controls, indicating no connection between this virus and acute respiratory illness [Feikin et al., 2012]. The absence of HPeV in the NPA samples in our study supports the observation that HPeV has no important role in the onset of respiratory diseases, specifically, bronchiolitis. However, our study population was quite young (<6 months) and was most likely still under the protection of maternal antibodies.

Furthermore, other potential respiratory illness-causing viruses were detectable in most of the samples. The presence of HPeV in MEF, NPA and other sample types suggests that HPeV is able to spread widely around the host body although not necessarily in connection to any specific disease.

4.6 Phylogeny of Finnish HPeV strains (I, IV)

Phylogenetically, the 78 Finnish HPeV isolates detected in this study predominantly cluster with other European strains (Figure 6). HPeV1 isolates from MEF of AOM-patients (IV) do not particularly diverge from most of the other HPeV1s isolated from healthy children (I). All of the Finnish HPeV1 isolates cluster together with more recently isolated HPeV1 strains (HPeV1B) rather than with the original HPeV1 reference strain Harris (HPeV1A). This finding suggests that the HPeV1 isolates circulating in Finland originated from a more recent HPeV1B lineage.

The HPeV3 from a child with suspected sepsis (FI110988) clusters together with the Finnish isolate from 2006. These two strains share sequence similarities to reference strains from the Netherlands, Canada and Germany. In contrast, one of the Finnish HPeV3 isolates from 1997 is more similar to a Japanese reference strain. The other Finnish HPeV3 isolate from 1997 is genetically most divergent from all of the other strains. HPeV6 isolates from 2001 are more closely related with American and Japanese strains, whereas another HPeV6 isolate from 2002 groups closer to the cluster formed by Brazilian and German strains.

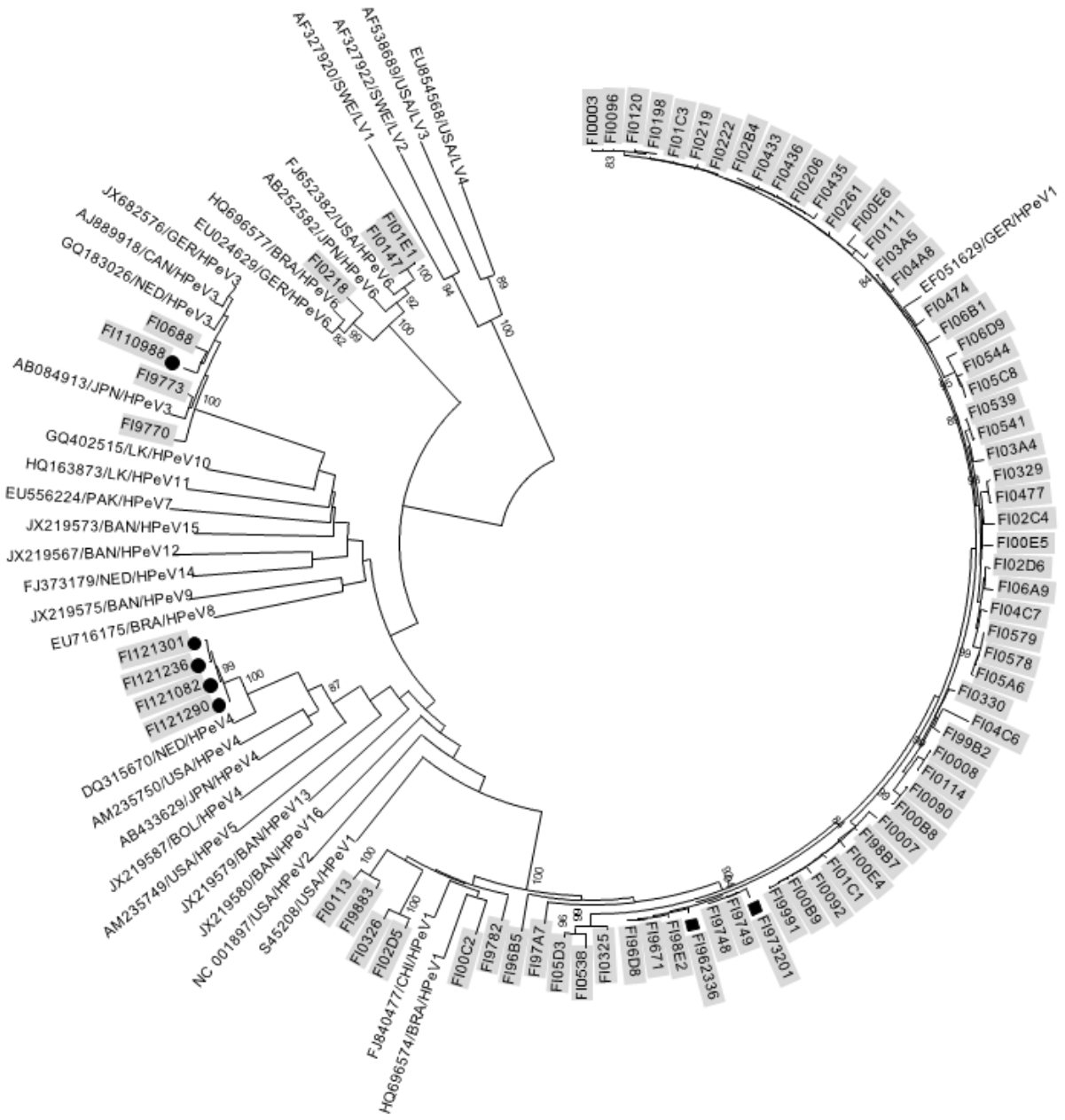

Figure 6 Evolutionary relations of 78 Finnish HPeVs (marked with a grey background) isolated in Studies I and IV, as well as reference strains from a 498 bp long region in VP1. Isolates from hospitalised children are marked with circles, whereas the ones from AOM patients are marked with squares. The country of discovery and the genotype are indicated for each reference strain.

The Finnish HPeV4 isolates shared high sequence homology, with 95% similarity in nucleotide and 98% in amino acid sequence, with the Dutch reference strain of HPeV4 (K251176-02), which was isolated in 2002 [Benschop et al., 2006a]. High sequence similarity to the reference strain in the VP1 area suggests that the genetic differences of the Finnish isolates, which have evolved into more potent strains than the reference strain, must be elsewhere in the genome. The determinants of picornavirus virulence may also reside outside the VP1 region. For poliovirus, the genetic markers affecting the virulence locate into 5'-UTR [Gutierrez et al., 1997; Westrop et al., 1989], whereas the factors affecting that of EV71 reside in the 5'-UTR and 2A, in addition to VP1 region [Li et al., 2011]. Therefore, a genome-wide genetic comparison between the HPeV4 reference strain and the Finnish HPeV4 isolates would deliver further insights regarding how the strains diverge on a molecular level.

The phylogenetically close relation between Finnish isolates and different HPeV strains from around the world underline the global nature of HPeV distribution. However, because the phylogenetic relations analysed in the present study are based on a relatively short sequence in the VP1 protein-encoding region, only a general observation can be made. As aforementioned, whole-genome sequence-based analysis would provide more information regarding the variations of these isolates and would reveal the potential large-scale changes caused by recombination events [Benschop et al., 2008c]. The evolution of parechoviruses is rapid, and recombination, which often causing large changes in HPeV genomes, is common [Benschop et al., 2008c; Calvert et al., 2010; Sun et al., 2012].

4.7 High detection rate of Ljungan virus-specific antibodies in Finland (V)

LV causes various diseases in its natural hosts, rodents, whereas its association with human diseases lacks evidence. To study the circulation of LV in Finnish human and rodent populations, we set up IFA tests to detect LV-specific antibodies. Cross-testing the LV IFA with LV microneutralisation and HPeV, which is the most closely related virus to LV, microneutralisation for types 1-6 assured the test's specificity, with no detected cross-reaction. The LV IFA test negative samples had high titres of nABs against HPeV types 1, 2 and 4 to 6, whereas antibodies against HPeV3 were absent in all samples. However, due to the differences in the microneutralisation methods, the titres of LV and HPeV were incomparable.

A set of human samples for LV IFA-testing was collected from humans with suspected Puumala virus-induced infection, who were, consequently, assumed to have likely been in contact with bank voles. The seroprevalence for LV was higher in humans (36%) than in bank voles (18%). This result is particularly surprising because bank voles are the natural host of LV and because the majority of LV strains thus far have been isolated from bank voles and from other related rodents [Niklasson et al., 1999; Salisbury et al., 2014]. Despite efforts, LV RNA has been detected in only few human samples [Niklasson et al., 2009b; Samsioe et al., 2009; Tapia et al., 2010], and isolating LV from these samples has been unsuccessful. Therefore, LV infection in humans and the LV-induced immunological reaction are poorly characterised.

The higher seroprevalence of LV in humans than in bank voles does not support the zoonotic relation suggested for LV. In contrast, our results suggest circulation of LV or of a LV-like virus among human populations. Another possibility is that some other common virus shares immunogenic epitopes with LV and is responsible for raising the antibodies in humans that are detectable with LV IFA. Studies that are more detailed are required to determine which virus is responsible for the presence of LV-specific nABs in humans.

5 Concluding remarks

The *Parechovirus* genus represents a relatively recent genus of viruses, including the human pathogens HPeVs and the rodent-infecting LVs. The presence of HPeVs among human populations is globally common and is often found in children with mild clinical symptoms. Increasing evidence has connected HPeVs to severe infections in neonates. LVs have been attributed with a potential zoonotic behaviour; however, their connection to human diseases remains to be established. Our project aimed to deepen the available knowledge regarding HPeV and LV by describing their epidemiology in Finland and by examining their involvement in various paediatric diseases.

The epidemiological observations in this study demonstrated that HPeVs are highly common in healthy Finnish children. Practically every individual encounters HPeVs during the first years of life. The common presence of HPeV1 in healthy children suggests that HPeV1 often participates in unapparent infections. In Finland, HPeV appears to be in circulation throughout the year, with a seasonal peak from October to November/December. Although antibodies against many HPeV genotypes were present, the genotype primarily detectable from stool samples was HPeV1. Therefore, future studies are required to examine the actual circulation of other types of HPeV.

Our microneutralisation data demonstrated that antibodies against HPeV1 to 6 are present in high levels in adults and children over five years old. Although the seroprevalence of HPeV-specific antibodies expectantly increased with population ages, this seroprevalence already reached near maximum in five-year-old children. The high seroprevalence for HPeV2 and 4 to 6 also indicated that these types are much more common than previously observed. Interestingly, all six types appear to be circulating in Finland. Only antibodies against HPeV3 were detectable in low levels. Intrigued by this fact, we observed that this genotype might employ a strategy to avoid its own neutralisation. Thus, studies based on HPeV3 antibodies do not deliver a reliable picture regarding its prevalence.

Because HPeV3 is a serious pathogen causing severe neonatal infections, the mechanism and role of its stronger pathogenicity demands further investigated.

The majority of HPeV-induced infections seem to be clinically mild or unapparent, thus remain undetected. Although HPeVs are extremely common in young children, we observed no connection between them and two common paediatric illnesses, AOM and bronchiolitis.

We detected and isolated HPeV type 4 for the first time in Finland from young children with sepsis-like disease during a potential local epidemic in autumn 2012 in Helsinki. The association of HPeV4 to severe human diseases had not been established before this study. These first severe HPeV-induced infections in Finland demonstrated that the role of HPeVs in cases of suspected CNS-infection or sepsis-like disease in young children is as serious as that of EV. Therefore, we stressed the addition of HPeV detection to clinical diagnostics. Specific knowledge regarding causative agents in neonatal infections is vital for ensuring the best course of treatment. Further investigations are required to understand why neonatal HPeV4 infections previously remained undetected. A complete sequence analysis of the Finnish HPeV4 strain may help in clarifying the recent epidemic in Finland. An analysis of maternal antibodies represents a further measure in resolving the question of whether children with no maternal antibodies are at higher risk for HPeV4 infection. An analysis of different sample pools from individuals of different ages would provide additional insight regarding the potential circulation of HPeV4 in a wider population.

The seroprevalence for LV was higher in humans than in bank voles, suggesting that zoonotic transmission of LV from bank voles to humans does not occur. The presence of antibodies for LV in humans indicates that an LV or LV-like virus is commonly circulating in Finnish populations, independent of those viruses circulating in rodent populations. The identification and isolation of an LV or LV-like virus from humans in the future is not only important for obtaining a better picture of their circulation pattern but also for understanding the character of a human-borne LV.

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