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# The 3-Dimensional Play of Human Parechovirus Infection; Cell, Virus and Antibody

BRENDA MIRANDE WESTERHUIS





**THE 3-DIMENSIONAL PLAY OF HUMAN  
PARECHOVIRUS INFECTION;  
CELL, VIRUS AND ANTIBODY**

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# The 3-Dimensional Play of Human Parechovirus Infection; Cell, Virus and Antibody

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# 1.

General introduction

## Classification and genome organisation

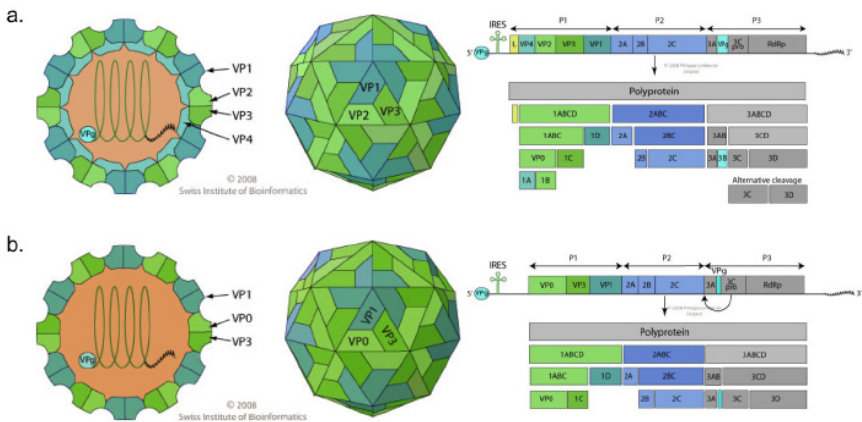
Human parechoviruses (HPEVs) are members of the family *Picornaviridae*. This is a large and diverse family of positive sense RNA viruses, containing several important human and animal pathogens. The picornaviruses are grouped in 26 genera: *Aphthovirus*, *Aquamavirus*, *Avihepatovirus*, *Avisivirus*, *Cardiovirus*, *Cosavirus*, *Dicripivirus*, *Enterovirus*, *Erbovirus*, *Gallivirus*, *Hepatovirus*, *Hunnivirus*, *Kobuvirus*, *Megrivirus*, *Mischivirus*, *Mosavirus*, *Oscivirus*, *Parechovirus*, *Pasivirus*, *Passerivirus*, *Rosavirus*, *Salivirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus* (1). The *Enterovirus* and *Parechovirus* genera include important causative agents of neurologic disease in humans. The genus *Enterovirus* is the biggest containing over 250 recognized types, including twelve species *Enterovirus A-J* and *Rhinovirus A-C*. These twelve species contain different serotypes including human rhinoviruses (HRVs), echoviruses (Es), coxsackie- A and B viruses (CAV and CBV) and the newer enterovirus types 68-121 (EVs). The species *Enterovirus A-D* contain all the human enteroviruses (HEVs). The genus *Parechovirus* is divided in the species Ljungan virus and Human Parechovirus, which contains only 16 recognized types.

The HPEV genome is ~7300 bases in length, and encodes a single polyprotein flanked by 5' and 3' untranslated regions (UTRs) (Figure 1). The covalently linked protein VPg (also designated 3B) is attached to the long 5'UTR of around 700 nucleotides preceding an open reading frame (ORF) of 2200 codons, followed by the 3'UTR of around 80 nucleotides and the poly(A) tail. Translation of the ORF, initiated by a cap-independent mechanism, is driven by an internal ribosome entry site (IRES), which results in a large polyprotein (2, 3). Picornavirus polyproteins are cleaved by virus-encoded proteases to give precursors and the final structural (capsid) and non-structural (NS) proteins. HPEVs together with kobuviruses have a different organisation of their structural proteins compared to other picornaviruses. The structural protein VP0, which is a precursor for VP4 and VP2 in most picornaviruses, is not cleaved; this VP0 maturation is thought to be critical for capsid stability. By that, HPEVs as well as Kobuvirus have only 3 structural proteins (VP0, VP3 and VP1) (4) (Figure 1). As in the other picornaviruses, the capsid is composed of 60 copies of each of the capsid proteins.

HPEVs non-structural 2A lacks the proteolytic activity seen in other picornaviruses, indicating incapability of the cleavage of VP1 and 2A proteins (5, 6). In the case of HPEVs, it seems that only one protease, 3Cpro, is involved in processing. Although it has been shown that HPEV1 2A binds to viral 3'UTR RNA, suggesting that 2A exerts an important function during HPEV1 replication, the exact function remains unknown (7). The 2C protein has been shown to have ATP hydrolysis and AMP kinase activities, which may contribute to

functions in the viral replication cycle. The 2B protein is an integral membrane protein that is mainly co-localized at the Golgi complex, mediating calcium release and increasing plasma permeability (8). Thereby co-localization with the ER is also observed. The 3A protein has also been observed to interfere with ER-to-Golgi transport. The function of the 3Dpol protein is well known as RNA dependent RNA polymerase.

Intra-species genome recombination is a well-known phenomenon among HEVs (9, 10). Analysis of full-length HPeV1-6 genomes has shown that the genomes between the different types are highly mosaic, showing type-specific clustering on the VP1, where this type-specific segregation is lost in the non-structural (NS) region 3Dpol. In case of HPeV3 this is different, where clustering is shown in both the VP1 and 3Dpol region of the different HPeV3 strains. Between HPeV1, -4, -5 and -6 strains recombination was frequently observed within the NS region, but only one HPeV3 sequence showed a loss of type-specific segregation (11, 12), which might suggest a different cell tropism.



**Figure 1.** Capsid structure and genome organization of HEV (a) and HPeV (b). [Source: ViralZone, SIB Swiss institute of Bioinformatics]

## Epidemiology of HPeVs

HPeVs are widespread pathogens, infecting mainly young children worldwide (13–15). The first HPeVs were discovered over 50 years ago during a diarrhoea outbreak in the USA (16), and were described as echovirus 22 and 23 in the *Enterovirus* genus, based on their HEV like cytopathic effect (CPE) in cell culture and non-pathogenicity in both mice and monkeys. However, based on evident differences in genome organization and biological

characteristics they were reclassified into their own genus and renamed as HPeV1 and 2 in 1999 (17–19). The US National Enterovirus Surveillance System at the Centers for Disease Control reported for the period 1970–2005 that HPeV1 and 2 accounted for a total of 1.8% of enterovirus detections (20). In addition, data extracted from monthly reports from all virological laboratories in Finland (between 1971–1992) showed a HPeV1 prevalence of 8% (21). From the US National Enterovirus Surveillance it was shown that between 1983 and 2005 73% of the HPeV1 and 68% of the HPeV2 infections occurred in children less than 1 year old (20). More extensive studies on HPeV1 revealed similar results; a Finnish follow-up study showed that at the age of 12 months 20% of the children experienced their first infection, and at the age of 36 months about 98% of the children had at least one HPeV infection (22). In a Norwegian longitudinal study it was observed that at 12 months of age 43% of the infants had experienced at least one HPeV infection, and at age 24 months 86% had encountered the virus (23).

Not until 2004, a third genotype was described, isolated from a stool specimen from a 1-year old child with transient paralysis (24). Since then, an additional 13 types have been described; a fourth type was identified in the Netherlands (25). Further phylogenetic analysis of previously isolated HPeV showed a fifth cluster within the genus. In 2007 a sixth type was isolated from a child with Reye's syndrome (26), and in 2008 several new types were characterized in Pakistan (HPeV7) (27), Brazil (HPeV8) (28), Thailand (Oberste *et al.*, unpublished) and the Netherlands (HPeV14) (29). Prevalence studies including new PCR-based detection methods and including the newer types HPeV3–6 showed high prevalence of HPeVs, and HPeV3 being the second most prevalent type. In a 3-year study period in the Netherlands a high prevalence of 16% was found by direct PCR screening of feces from hospitalized children under the age of 5 years (29). In feces from healthy children in Finland a prevalence of 6.4% was found (30). In both countries the circulation of HPeV1 is yearly prominent. In the Netherlands and confirmed by a study in the United Kingdom a clear periodicity is found for HPeV3 with seasonal peaks in 2000, 2002, 2004, 2006 and 2008 (29, 31–33), occurring mostly late summer.

Most studies show prevalence of HPeVs based on cell culture and PCR, but seroprevalence data remain rare. The available seroprevalence data do show that HPeVs are very common. For HPeV1 two Finnish studies showed seroprevalences of >97% in adults, and of the 21 neonates tested 95% had aHPeV1 antibodies (Abs), which are most likely maternal (22, 34). A Japanese study showed HPeV3 seroprevalence among women of childbearing age to be 68% (24). A second Japanese study showed antiHPeV3 Abs present in a group of 22 adults after infection with HPeV3 (86%), however, only low neutralizing titers were found up to 71 days after onset of illness (35). Seroprevalence data for the other HPeV types are

not available. Determining seroprevalence can give a good insight on virus circulation and the existing immunity within different populations, which is important for understanding epidemiology and outbreaks.

### **Clinical aspects and diagnosis of HPeV infections**

The transmission route of HPeVs remains to be elucidated, but as for the closely related HEVs, transmission seems to occur via the fecal-oral and respiratory routes. HPeV are associated with a wide range of clinical symptoms from mild disease such as gastro-enteritis and respiratory tract infections to a variety of severe disease like myocarditis, sepsis-like illness, paralysis, encephalitis and meningitis. For HPeV1 infection diarrhoea is the most common symptom followed by respiratory symptoms (17, 34, 36). In an evaluation of the clinical picture of children with an HPeV4, 5 or 6 infection the main symptoms were gastro-intestinal and respiratory symptoms as well (37). Occasionally HPeV1, 4, 5 and 6 have been associated with severe disease (38–40). A recent study showed two cases of neonatal sepsis suspected to be caused by HPeV4, which was not shown before for this genotype (41). Although most severe disease cases associated with HPeV infection are caused by the HPeV3 genotype (24, 31, 42–46). A study on the clinical relevance of HPeV1 and HPeV3 detection in stool showed that a HPeV3 positive stool sample is commonly associated with clinical disease, whereas in case of HPeV1 there was only clinical relevance in case of underlying disease (36). Several reports showed that HPeV3 is the predominant type detected in cerebrospinal fluid (CSF), showing the importance of HPeV3 in CNS infections (31, 32, 47–50). In a big screening of CSF samples in the United Kingdom HPeV3 was even identified as the most common picornavirus type (32). A recent study showed that seizures and CNS symptoms were more common among the HPeV infected children compared to those infected with HEV, both virus positive in CSF (51). Additionally, HPeV3-infected children are generally below 6 months of age (median age 1.3 months), which is significantly younger than HPeV1-infected children who are mostly around 6 months (median age 6.6 months) (29, 33, 52). Although HPeV3 infections have been mostly detected in young infants in Europe and the US, in 2008 a symptomatic HPeV3 outbreak was reported in Japan with 22 cases among adults, suffering from myalgia, muscular weakness, sore throat and orchiodynia (35, 53).

The classical method for diagnosis of infection with HEVs and HPeVs has been virus isolation in cell culture from different clinical specimens. Difficulties in cell culture of HPeVs, however, have been reported (54). HPeV1 can be cultured on various cell lines, while HPeVs 3-6 induce only a low cytopathic effect (CPE) on a limited number of cell lines (26, 54, 55). For isolation of HPeV1, HPeV3 and HPeV6 from clinical samples, Watanabe *et*

*al.*, used 8 different cell lines (26). For HPeV3 it was shown that initial culturing of 3 clinical specimen showed induction of CPE on the LLCMK2 cell line after 14-18 days, albeit after passaging the virus to Vero cells, CPE appeared after 4-5 days (43). Benschop *et al.*, showed that the HT29 cell line is an efficient cell line to propagate most HPeVs from clinical samples except for HPeV3, which could only be isolated on A549 and Vero cells (54). Nowadays for the detection of picornaviruses in clinical specimens reverse transcriptase PCR (RT-PCR) is shown to be faster and more sensitive than cell culture (54). PCRs targeting the 5'UTR are commonly used to diagnose HPeV infection. Since the 5'UTR is highly conserved all HPeV types can be detected, and this method is highly sensitive (42, 56). Typing can subsequently be performed on the clinical sample by sequencing of the VP1 region (56, 57).

HPeV infections are now recognized as clinically relevant and highly sensitive and fast methods are implemented for detection. However, antiviral treatment is still not available. Successful vaccines have been developed against poliovirus, hepatitis A virus, the veterinary virus foot and mouth disease virus (FMDV), and recently against EV71 (58, 59). The development of antiviral therapy against HEVs and HPEV has not yet been successful. Therefore treatment options are limited and therapy is mainly supportive. One option for treatment is the use of intravenous immunoglobulins (IVIg); one blinded randomized controlled study showed that only neonates receiving IVIg with high specific Ab titers (>1:800) were able to clear the HEV (55). Despite these occasional positive results, treatment with IVIg is non-specific and efficacy has not been proven (60). A promising drug in HEV treatment was the capsid inhibitor pleconaril, showing clinical activity in some patient groups, and a favourable safety profile (reviewed in 60–62). Concerns about possible drug interactions and resistance resulted in rejection by the US FDA, and production was abandoned. HPeV1 and HPeV3 were resistant against this capsid inhibitor (64). Next to capsid inhibitors another promising option for antiviral therapy are compounds targeting the viral factors, like protease or polymerase inhibitors. A drug with wide anti-picornaviral activity would be preferable, and given the high clinical relevance of HPeVs they should be included into the test panels for antiviral compound development.

### **The role of the RGD motif in infection**

Sequence analyses of the HPeV genome revealed that the C-terminus of VP1 contains the arginine-glycine-aspartic acid (RGD) motif. This motif is conserved in HPeV1, 2, 4, 5 and 6, while HPeV3 and the newer identified HPeV7-16 lack this motif (1). The RGD motif has been shown to bind to the  $\alpha v\beta 3$ ,  $\alpha v\beta 6$  integrins as their receptors (65–68), resulting in virus entry into the host cell by a clathrin-dependent endocytic pathway (67). For HPeV1 it has been shown that viral infection could be blocked by RGD containing peptides or

by removal of the RGD motif from the HPeV1 genome (4, 65). The lack of this motif in HPeV3 and HPeV7-16 implies different receptor usage and mechanism of entry, possibly related to a different tropism, but these have not yet been determined. The RGD motif is also found in the enteroviruses CAV9 and E9 as well in FMDV (Aphthovirus genus), and is shown to play a role in attachment and entry (69–71). For CAV9 it has been shown that removal of the RGD motif is not fully inhibiting viral infection (72), showing an efficient RGD independent entry process of CAV9 (73). The RGD containing strain E9 strain was shown to be pathogenic for newborn mice, while the RGD lacking strains were not (71, 74–76). In case of CAV9, mutants without an RGD motif were found to be less pathogenic (77, 78). Both studies showed that pathogenicity of these strains was different in older mice. In case of E9 a paralytic response shown in newborn mice remained absent in older mice (74). Therefore it seems that for CAV9 and E9 RGD loss is associated with loss of pathogenicity, in contrast to what is found for HPeV3 being the most pathogenetic HPeV up to now. In HEVs major neutralizing antigenic sites reside on the VP1 capsid protein and to a lesser extent on VP2 and VP3 (79–81). For the RGD containing HEVs CAV9 and FMDV, the interaction of the antibody with the RGD motif is important for virus neutralization (80, 82). This antigenic site has also been shown to be important for HPeV1, by blocking of infection with RGD containing peptides (4, 83). Full neutralization was obtained with rabbit antiserum against VP1, but antiserum against VP0 could inhibit infection up to 80% as well (84). Peptide scanning of the viral capsid proteins with immune serum from an HPeV1 immunized rabbit, revealed several immunogenic sites. The rabbit immune serum clearly showed immunogenic reactivity with a site in the N-terminus of VP0 (aa82-99), and two regions in the VP3 capsid protein, one located at the N-terminus of VP3 (aa15–35) and the other at the C-terminal region (aa183–195) (83). Antisera raised against the RGD containing peptide was able to neutralize 51% of HPeV1 infection, and the antisera raised against the VP0 peptides neutralized 43% of HPeV1 infection (83), indicating that HPeV1 contains several antigenic sites. These VP0 and VP3 antigenic sites for HPeV1 have never further been characterized, nor are the antigenic sites of the other HPeV genotypes.

### **Humoral immunity and antibody protection**

The immune response against picornaviruses is mainly humoral, and is supposed to be type specific. The humoral immune response involves antibodies (Abs) produced by B cells, that prevent further spread of the infection by 1) coating the virus and preventing it from entering the cells, 2) by coating the surface to enhance phagocytosis, or 3) by complement activation. The role of the humoral immune system in defence against picornavirus infections is illustrated by the vulnerability of newborns and patients with humoral immune



deficiencies for severe HEV infections, while T cell deficient patients do not seem to experience problems with these infections (64). During pregnancy IgGs are transported over the placenta, resulting in high Ab levels protecting the child until the newborn can produce its own Abs. This Ab protection will last up to a several months. Failure in Ab protection due to the absence of specific Abs may lead to severe disease and even to death. In neonates with severe HEV infection, the maternal Ab titers against the specific HEV serotype were shown to be absent or low (85, 86).

Patients with a deficient humoral immune response, such as X-linked agammaglobulinemia, are at great risk of chronic enteroviral infections (87). In immunodeficient patients prolonged poliovirus replication was shown (88). In patients with agammaglobulinemia, coxsackievirus (CV) infection could spread to and stay persistent in the central nervous system (89–91). For CV it has been shown that the humoral immune response plays a prominent role in limiting virus spread and in viral clearance (92); CBV3 infection in B cell deficient mice results in chronic infections with high viral titers (93). The role of the humoral immune response seems to be clear, but the role of the T cell response in CV infections is not clear, showing discordant data with different strains and in mice (92, 94–96). As antiviral drugs against HEV are currently lacking, IVIg is often used as a replacement therapy in patients with a primary humoral immune deficiency, preventing chronic enteroviral infections (87, 97, 98). IVIg administration remains an unspecific treatment against HEV infections, and it cannot protect against lowly circulating or newly introduced HEV or HPeV strains against which there is no immunity in the population. A more targeted option for antiviral treatment of HEV and HPeVs would therefore be the use of specific monoclonal Abs. New approaches for generating human monoclonal Abs have been successful against influenza virus (99) and respiratory syncytial virus (100). For HEVs neutralizing Abs are supposed to be type specific, which makes cross-neutralization probably rare among over a 100 serotypes, and the development of monoclonal Abs against so many types is unattractive. In contrast, the HPeV group is small, depicting high similarity among the different genotypes, whereas several antigenic epitopes are described and cross neutralization is observed (83, 84). This would make HPeVs an ideal target for development of monoclonal Ab (mAb) treatment.

To achieve this, localization of antibody binding sites is important for obtaining information concerning the mechanism of neutralization and receptor binding. For HRV2, HRV14 and FMDV virus Ab neutralization is extensively studied using cryo-electron microscopy (cryo-EM) (101–107). For Ab neutralization of FMDV the VP1 C-terminal loop is important, where direct interaction is shown between the RGD motif and several complementary regions of the Ab molecule (82). Structural 3D reconstructions of HPeV1 by Cryo-EM revealed a typical pseudo T=3 organization seen in picornaviruses, and confirmed the

binding of  $\alpha\beta_3$ ,  $\alpha\beta_6$  integrins to the RGD loop of HPeV1 VP1 (68). Ab neutralization of HPeV1 has never been studied using these techniques. For HPeV3 the 3D virus structure has not yet been revealed. Structural studies are important to gain more insight in the differences between HPeV1 and HPeV3, leading to better understanding of receptor usage and Ab binding, and thus of the pathogenic difference between type 3 and other HPeVs.

## Thesis outline

HPeV3 has been shown to be the ‘odd one out’ compared to the other genotypes; HPeV3 is more often associated with severe disease, infects significantly younger infants, seems to be more difficult to culture, and does not recombine with other HPeV types. The aim of this thesis is to study whether these differences could be explained by differences in viral tropism and Ab protection between the subsequent HPeV types.

The first part of the thesis focuses on differences in viral tropism among HPeV types. In **Chapter 2**, we describe the growth characteristics of the different genotypes on different cell lines. To more extensively look into the differences between HPeV3 and HPeV1 related to the clinical outcome of infection, we describe the specific cell tropism and neutralization of human parechovirus types 1 and 3 in **Chapter 3**. To study the importance of the human airway as a primary replication site of HPeVs and differences in virus tropism, we determined replication kinetics of different HPeVs types in a human respiratory primary cell culture system (HAE) (**Chapter 4**).

The second part of the manuscript focuses on neutralization of HPeVs. **Chapter 5** describes the seroprevalence of neutralizing (protective) Abs among different groups in Finland and the Netherlands. The (cross-) neutralization of HPeVs by different HPeV1 and HPeV3 polyclonal and monoclonal Abs is extensively described in **Chapter 6 and 8**. Using cryo-EM, we determined the different neutralizing epitopes for HPeV1 (**Chapter 7**) and we revealed a high-resolution structure of HPeV3 (**Chapter 9**).

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# 2.

## **Growth Characteristics of Human Parechovirus 1 to 6 on Different Cell Lines and Cross- neutralization of Human Parechovirus Antibodies; a Comparison of the Cytopathic Effect and Real Time PCR**

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## Abstract

**Background:** Human parechoviruses (HPEVs) are among the most frequently detected picornaviruses in humans. HPEVs are usually associated with mild gastrointestinal and respiratory symptoms with the exception of HPEV3 which causes neonatal sepsis and CNS infection. Previous studies showed various results in culturing different HPEV genotypes, inducing only a low cytopathic effect (CPE).

**Methods:** *In vitro* growth characteristics of the different HPEV genotypes in a range of 10 different cell lines are scored with CPE and measured in the supernatant by real time PCR. In the optimal cell line for each genotype a standard neutralization assay with the available HPEV antibodies (Abs) was performed and scored by CPE and measured by real time PCR.

**Results:** All six HPEV types were able to replicate on the RD99, A549, and Vero cell lines. HPEV1 was the only genotype able to replicate on all cell lines. Most efficient growth of HPEV1, 2, 4, 5, and 6 was shown on the HT29 cell line, while HPEV3 was unable to replicate on HT29. In all cases viral replication could be measured by real time PCR before CPE appeared. The polyclonal Abs available against HPEV1, 2, 4 and 5 all showed neutralization of their respective genotype after 7 days with inhibition of >60% in real time PCR and full inhibition of CPE, although cross- neutralization is shown. Replication of HPEV3 could only be inhibited by 12% by the anti-HPEV3 (aHPEV3) Ab and no inhibition of CPE was shown after 7 days.

**Conclusion:** When replication is monitored by PCR, growth of HPEV genotypes 1 to 6 is supported by most of the cell lines tested, where viral replication is measured before appearance of CPE. A combination of HT29 and Vero cells would therefore support replication of all culturable HPEV types, so viral replication could be detected by PCR within 3 days for all genotypes.

In addition, we showed efficient neutralization for HPEV1, 2, 4, 5, while cross- neutralization was shown between these types, indicating possible common neutralizing epitopes. For HPEV3 no efficient (cross-) neutralization was shown, indicating different neutralizing epitopes for HPEV3 compared to the other HPEV genotypes.

**Keywords:** Parechovirus, Replication, Cross- neutralization, Real time PCR, CPE

## Introduction

Human Parechoviruses are single stranded RNA viruses belonging to the *Picornaviridae* family. HPeVs are associated with a wide range of clinical manifestations ranging from gastrointestinal and respiratory symptoms to more severe symptoms like central nervous system (CNS) infections and neonatal sepsis [1-4]. Nowadays, 16 genotypes are known [5-10], HPeV1 and HPeV2 were first isolated in the 1950's, whereas the third type was only described in 2004. This late discovery of HPeV could be due to difficulties of HPeVs detection in cell culture, because of only a low induction of CPE, most pronounced for HPeV3 and HPeV6. HPeV7 to 16 have not been cultured in standard cell lines used in diagnostics. Previous studies showed various results in culturing different HPeV genotypes. HPeV1 can be cultured on various cell lines such as BSC-1, Caco2, RD-18S, Vero, LLCMK2, RD99, HT29, tMK, and A549, while other newer HPeVs induce only a low cytopathic effect (CPE) on a limited number of cell lines [7,11,12]. For isolation of HPeV1, HPeV3 and HPeV6 from clinical samples, Watanabe *et al.*, used 8 different cell lines [7]. For HPeV3 it was shown that initial culturing of 3 clinical specimen showed induction of CPE on the LLCMK2 cell line after 14–18 days, albeit after passing the virus to Vero cells, CPE appeared after 4–5 days [1]. Benschop *et al.*, showed that the HT29 cell line is an efficient cell line to propagate most HPeVs from clinical samples except for HPeV3, which could only be isolated on A549 and Vero cells [12]. None of the HPeVs could be detected by growth on the HEL cell line, a cell line that is regularly used to culture enteroviruses (EVs). In addition it was shown that only 42% of clinical samples positive for HPeV by PCR could be cultured, using 6 different cell lines [12]. With difficulties in culturing HPeVs, there is also a limitation in isolation of all the different strains for usage in HPeV serotyping assays. Because HPeV detection with cell culture is laborious and limited to HPeV1-6, PCRs targeting the 5'UTR are commonly used to diagnose HPeV infection [12-15]. Since the 5'UTR is highly conserved all HPeV types can be detected, and this method is highly sensitive. However, cell culture is still used as a diagnostic method in laboratories worldwide. In addition, cell culture is imperative to obtain virus isolates for further studies.

Although cell culture is a commonly used method in diagnostics and research, HPeVs are difficult to culture for many laboratories (personal communication). HPeVs culture characteristics have never been completely elucidated in cell culture experiments. In this study we investigated *in vitro* growth characteristics of different HPeV genotypes by measuring viral RNA in the supernatant of a range of cell lines by real time PCR. In addition, neutralization capacities of available type-specific antibodies is tested in *in vitro* cell culture.

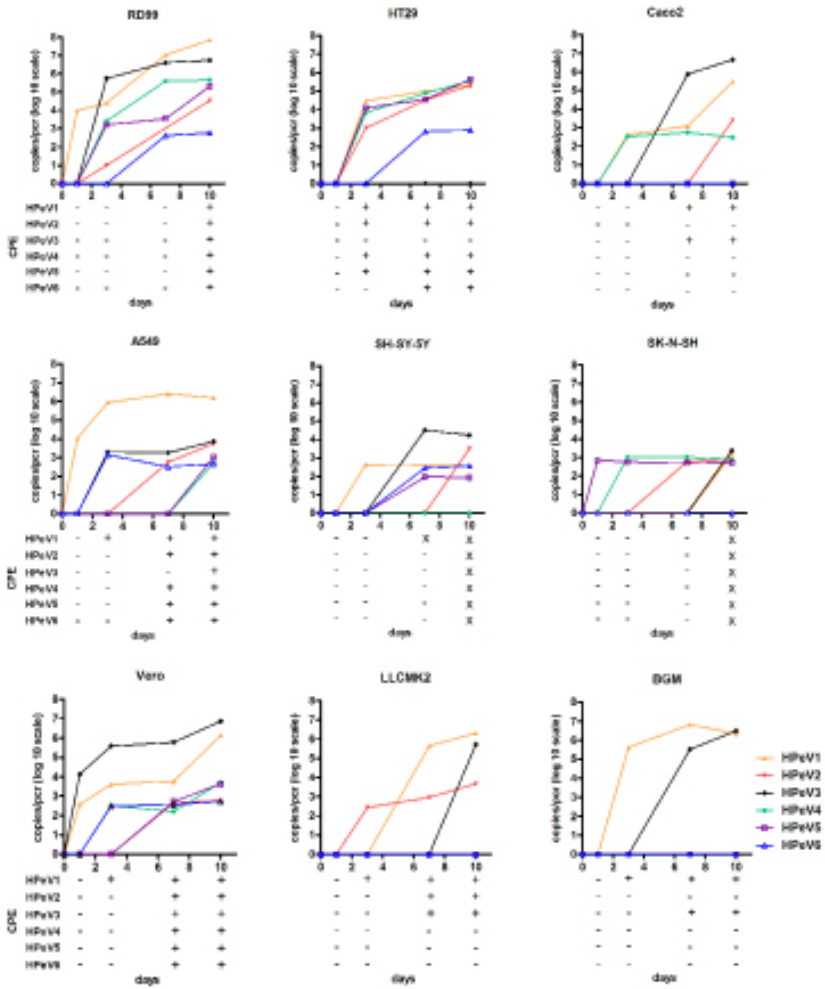
## Results

### Replication of HPeV1 to 6 on different cell lines

Virus growth characteristics of HPeV1 to 6 were determined on HT29, Caco-2, A549, RD99, Hep-2, Vero, BGM, LLCMK2, SK-N-SH, and SH-SY-5Y by infection with a fixed MOI (0.001). Replication was monitored in the culture supernatant at day 0, 1, 3, 7 and 10 by quantitative RT-PCR, and CPE was scored at the same time points.

All six HPeV types were able to replicate on the RD99, A549, and Vero cell lines (Figure 1, left panel). HPeV1 was able to replicate on all cell lines. Rapid replication kinetics reaching high virus titers were found on A549, RD99 and BGM cells. Rapid replication kinetics with lower titers were found on HT29, Caco-2 and LLCMK2, while replication kinetics were slow on SH-SY-5Y and SK-N-SH cells. HPeV2 showed good replication on the HT29 cells, no replication on the BGM cell line and only slow replication kinetics on the other cell lines reaching low titers. HPeV3 reached high titers on Vero, RD99, and Caco-2 cells but could also replicate on SH-SY-5Y, SK-N-SH, LLCMK2, A549, and BGM. HPeV3 was unable to replicate on HT29, while this cell line supported the growth of all other HPeV types. HPeV1 and 3 were the only two strains able to replicate on BGM cells. Replication kinetics of types HPeV4 to 6 on the cell lines supporting their growth were mostly slower and/or reached lower virus titers than for HPeV1 or 3. HPeV4 and 5 showed good replication on the HT29 and RD99 cells, and only low replication on the A549, Caco-2 (HPeV4), SH-SY-5Y (HPeV5), SK-N-SH and the Vero cells. HPeV6 showed inefficient replication: only slow replication was found in A549, RD99, Vero, HT29 and SH-SY-5Y. None of the HPeV types were able to replicate on Hep-2 cells (data not shown). On the Caco-2 cells CPE induction was limited to HPeV1 and 3, appearing in 7 to 10 days, while viral replication of HPeV2 and 4 could also be shown within 6 days by real time PCR. For HPeV3 CPE appears after 7 days only on the Vero cell line, while viral replication could be measured within one day with PCR.

Overall, replication of HPeV1 to 6 was observed in most cell lines including neural cell lines, although with different kinetics. With respect to replication on the human-derived cell lines, differences were most pronounced in the A549 with highest growth kinetics of the HPeV1, and in the HT29 where all genotypes could replicate except HPeV3. There was no cell line exclusively supporting replication of a specific HPeV genotype.



**Figure 1.** Replication kinetics of HPeV1 to 6. Growth kinetics of laboratory adapted HPeV1 to 6 on different cell lines including appearance of CPE at days 0, 1, 3, 7 and 10. Cells were infected with HPeVs at a MOI 0.001 and viral RNA was detected in the supernatant with RT-PCR at days 0, 1, 3, 7 and 10. The 10log virus copies were calculated with a standard curve, and the input virus copies per PCR at day 0 was subtracted. HPeV1: orange line, HPeV2: red line, HPeV3: black line, HPeV4: green line, HPeV5: yellow line, HPeV6: blue line. CPE was scored as positive (+), negative (-) and dead cells (X).

## Neutralization capacity

Polyclonal Abs available against HPeV1 to 5 were tested for neutralization capacity against HPeV1 to 6 by inhibition of CPE in cell culture (Table 1) and the inhibition was measured by real-time PCR (Table 2) at day 3 (Table S1,S2) and day 7 (Table 1,2). Only the aHPeV2 Ab fully neutralized its respective genotype without any cross-neutralization. The aHPeV1 Ab showed substantial cross-neutralization, with the HPeV4 and HPeV5 prototypes showing 60% inhibition of replication and reduced CPEs from 4+ to 2+. The aHPeV1 Ab also inhibited replication of HPeV6 by 27%, showing a reduction in CPE from 4+ to 3+. The aHPeV4 Ab inhibited replication of both HPeV4 and HPeV5 to the same extent (56-60%) with full inhibition of CPE formation, while the minor replication inhibition up to 16% for the other HPeV types was not visible by reduction of CPE. The aHPeV5 Ab showed high cross-neutralization with HPeV1 and lower with HPeV4 and HPeV6.

The aHPeV3 Ab inhibited replication of our laboratory prototype HPeV3-150237 only by 12%, while no reduction of CPE was shown after 7 days. Culturing for 3 or 7 days before measuring replication by PCR did not make a difference (Table S2).

**Table S1.** Neutralization of HPeV1 to 6 by polyclonal Abs, read out of CPE at day 3 post infection.

	CPE score Day 3					
	aHPeV1-Ab <sup>2</sup>	aHPeV2-Ab <sup>2</sup>	aHPeV3 Ab <sup>2</sup>	aHPeV4 Ab <sup>2</sup>	aHPeV5 Ab <sup>2</sup>	No Ab
<b>HPeV1- Harris<sup>1</sup></b>	- *	2+	3+	1	-	2+
<b>HPeV2- 751312<sup>1</sup></b>	1+	-	1+	1+	1+	1+
<b>HPeV3- 150237<sup>1</sup></b>	1+	1+	1+	1+	1+	1+
<b>HPeV4- 251176<sup>1</sup></b>	-	3+	3+	-	-	3+
<b>HPeV5- 552322<sup>1</sup></b>	-	3+	3+	-	-	3+
<b>HPeV6- 550389<sup>1</sup></b>	-	2+	2+	2+	-	2+

<sup>1</sup>100TCID50 infection on HT29 (HPeV1, 2, 4, 5) and Vero (HPeV3) cells

<sup>2</sup> Ab dilution 1:100

\* - no CPE; 1+ 0- 25% CPE; 2+ 25-50% CPE; 3+ 50-75% CPE; 4+ 75-100% CPE

**Table S2.** Neutralization of HPeV1 to 6 by polyclonal Abs, percentage of inhibition measured by real time PCR at day 3 post infection.

	Percentage inhibition in real-time PCR Day3				
	aHPeV1-Ab <sup>2</sup>	aHPeV2-Ab <sup>2</sup>	aHPeV3 Ab <sup>2</sup>	aHPeV4 Ab <sup>2</sup>	aHPeV5 Ab <sup>2</sup>
<b>HPeV1- Harris<sup>1</sup></b>	100	2	0	11	100
<b>HPeV2- 751312<sup>1</sup></b>	0	100	0	16	26
<b>HPeV3- 150237<sup>1</sup></b>	10	6	12	6	13
<b>HPeV4- 251176<sup>1</sup></b>	60	0	0	60	47
<b>HPeV5- 552322<sup>1</sup></b>	60	0	0	56	43
<b>HPeV6- 550389<sup>1</sup></b>	27	0	0	2	33

<sup>1</sup> 100TCID<sub>50</sub> infection on HT29 (HPeV1, 2, 4, 5) and Vero (HPeV3) cells

<sup>2</sup> Ab dilution 1:100

**Table 1.** Neutralization of HPeV1 to 6 by polyclonal Abs, read out of CPE at day 7 post infection

	CPE score Day 7					
	aHPeV1-Ab <sup>2</sup>	aHPeV2-Ab <sup>2</sup>	aHPeV3 Ab <sup>2</sup>	aHPeV4 Ab <sup>2</sup>	aHPeV5 Ab <sup>2</sup>	No Ab
<b>HPeV1- Harris<sup>1</sup></b>	- *	4+	4+	4+	-	4+
<b>HPeV2- 751312<sup>1</sup></b>	4+	-	4+	4+	4+	4+
<b>HPeV3- 150237<sup>1</sup></b>	4+	4+	4+	4+	4+	4+
<b>HPeV4- 251176<sup>1</sup></b>	2+	4+	4+	-	1+	4+
<b>HPeV5- 552322<sup>1</sup></b>	2+	4+	4+	-	-	4+
<b>HPeV6- 550389<sup>1</sup></b>	3+	4+	4+	4+	2+	4+

<sup>1</sup> 100TCID<sub>50</sub> infection on HT29 (HPeV1, 2, 4, 5) and Vero (HPeV3) cells

<sup>2</sup> Ab dilution 1:100

\* - no CPE; 1+ 0- 25% CPE; 2+ 25-50% CPE; 3+ 50-75% CPE; 4+ 75-100% CPE



**Table 2.** Neutralization of HPeV1 to 6 by polyclonal Abs, percentage of inhibition measured by real time PCR at day 7 post infection.

	Percentage inhibition in real-time PCR Day7				
	aHPeV1-Ab <sup>2</sup>	aHPeV2-Ab <sup>2</sup>	aHPeV3 Ab <sup>2</sup>	aHPeV4 Ab <sup>2</sup>	aHPeV5 Ab <sup>2</sup>
<b>HPeV1- Harris<sup>1</sup></b>	85	6	7	6	84
<b>HPeV2- 751312<sup>1</sup></b>	8	80	1	5	2
<b>HPeV3- 150237<sup>1</sup></b>	2	0	12	5	5
<b>HPeV4- 251176<sup>1</sup></b>	39	0	0	64	14
<b>HPeV5- 552322<sup>1</sup></b>	58	0	0	54	63
<b>HPeV6- 550389<sup>1</sup></b>	28	0	1	0	30

<sup>1</sup>100TCID50 infection on HT29 (HPeV1, 2, 4, 5) and Vero (HPeV3) cells

<sup>2</sup> Ab dilution 1:100

## Discussion

This study shows different replication kinetics of the culturable HPeV genotypes 1 to 6 on a set of different cell lines with real time PCR. Previously, difficulties have been encountered in culturing HPeVs, especially for HPeV3 and HPeV6 [1,7,12], which could only be propagated on a limited number of cell lines showing poor production of CPE. Replication monitored by PCR shows that growth of HPeV genotypes 1 to 6 was supported by most of the cell lines tested, and by comparing replication kinetics measured by PCR and seen by CPE, viral replication can be measured before CPE appears in the infected cell line, while sometimes CPE does not occur at all. In three of the nine cell lines (Vero, RD99 and A549) all six prototypes could be propagated. All HPeV1 to 6 genotypes show replication with high viral titers on the RD99 cell line, but CPE is hardly seen. The combination of HT29 and Vero cells is suitable to detect all culturable HPeV types by CPE, where viral replication could be detected with PCR within 3 days for all genotypes. By measuring replication by PCR we showed that HPeV6 was able to replicate on 5 of the 9 cell lines tested and HPeV3 on all cell lines except HT29. In contrast, HPeV3 was able to replicate on the Caco-2 gastrointestinal cell line. This difference can be due to the fact that Caco-2 cells are known for their ability to differentiate to a more fetal-like phenotype rather than adult ileal enterocytes, resulting in different receptor expression [16,17]. Between the different genotypes, replication kinetics

were found to differ between cell lines, however there was no cell line exclusively supporting replication of a specific HPeV genotype. All cell lines used in our study are continuously growing cell lines obtained from tumours that do not necessarily represent the tissue from which they originated. To get better representation of the *in vivo* replication of the different genotypes, primary cell systems need to be set up.

In our study we showed that the available HPeV Abs neutralized their respective genotype with high percentages of inhibition after 7 days. Thereby we show that inhibition of viral replication can already be measured after 3 days with similar inhibition percentages, while CPE is only starting at that time for some genotypes. Measuring neutralization by reduction of CPE or replication inhibition by PCR is comparable: inhibition of replication > 60% showed full reduction of CPE after 7 days. Inhibition between 20-60% gives reduction of 1+ or 2+ of CPE and inhibition of <20% shows no reduction of CPE. By PCR as well as by CPE read out, we showed cross neutralization of presumably type-specific polyclonal Abs, while neutralizing Abs for picornaviruses are considered to be serotype-specific. However, cross-neutralization has been reported before as shown in two studies with CAV9 and HPeV1 [18,19]. For EVs the main immunogenic region is the capsid protein VP1. In both CAV9 and HPeV1 the C-terminus of the capsid protein VP1 contains the arginine- glycine- aspartic acid (RGD), which has been shown to be an important antigenic site. Although by peptide scanning a highly immunodominant epitope has been recognized in the N-terminal region of the VP0 capsid, of which the neutralizing Abs against VP0 showed high reactivity with HPeV1. Abs raised against these different antigenic sites possibly differ in their ability to cross- neutralize HPeV infections. Reinfections in children aged 0-3 years are reported, showing a lower incidence of a second infection and infections within the first year after the initial infections were rare [20,21]. This lower incidence of a second infection in children is possibly due to partial cross- neutralization. Thereby HPeV infections in adults are hardly found.

For HPeV, the available Abs are all polyclonal obtained from immunization of animals, hence the presence of Abs that can bind to common epitopes is expected. Generating seroprevalence data this cross- neutralization should be taken in account. As we have shown before, the available aHPeV3 Ab A308-99 was not able to neutralize our HPeV3-150237 strain despite efficient binding [22]. The lack of neutralisation of HPeV3 with the Japanese Ab could indicate that the antibody has partly lost its potency. However, previously we showed that after HPeV3 infection in two different donors the obtained sera did not neutralize HPeV3. Further research is needed to confirm whether HPeV3 is indeed difficult to neutralize, or whether these results are based on *in vitro* artefacts such as a defect antibody or the influence of the cell line used for *in vitro* neutralization assays. It could be that the

HPeV3 virus structure does not permit Abs to reach the neutralizing epitope, but more research is needed to elucidate the mechanism of HPeV3 neutralization.

In summary, we showed that when replication is monitored by PCR, growth of HPeV genotypes 1 to 6 is supported by most of the cell lines tested. Viral replication could be measured before CPE appeared in the infected cell line. For HPeV1, 2, 4, 5 and 6 neutralization is shown with high inhibition of viral replication; with cross neutralization shown, in contrast the available HPeV3 Ab shows almost no (cross-) neutralization. More research needs to be done to elucidate the differences between HPeV3 and the other genotypes.

## Materials and Methods

### Cell lines

For virus culture, the following cell lines were used: human colon carcinoma (HT29), human colon adenocarcinoma (Caco-2), human lung carcinoma (A549, kindly provided by the University Medical Center, Leiden), rhabdomyosarcoma (RD99), epidermoid carcinoma of the larynx (Hep-2), African green monkey kidney (Vero), buffalo green monkey kidney (BGM, kindly provided by Dr. van Kuppeveld, St. Radboud University, Nijmegen), rhesus monkey kidney (LLCMK2, kindly provided by the Municipal Health Services, Rotterdam), and human neuroblastomas (SK-N-SH, kindly provided by Dr. Scheper, department of Neurogenetics, Academic Medical Center; SH-SY-5Y, kindly provided by Dr. Tauriainen, University of Tampere, Finland). The cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with L-glutamic acid (0.2X), non essential amino acid (1X), streptomycin (0.1 µg/ml) and ampicillin (0.1 µg/ml). For HT29, A549, RD99, Hep-2, Vero, LLCMK2, and BGM the medium was supplemented with 8% heat-inactivated Fetal Calf Serum (FCS) and for the Caco-2 cell line with 20% heat-inactivated FCS. The human neuroblastoma cell lines were cultured in Dulbecco's MEM and supplemented with 10% heat-inactivated FCS, L-glutamic acid (0.2X), non essential amino acid (1X), streptomycin (0.1 µg/ml) and ampicillin (0.1 µg/ml).

### Virus strains/ Virus cultivation

The following HPeV strains were used as prototypes: HPeV1A Harris, HPeV2-751312, HPeV3-150237, HPeV4-251176, HPeV5-552322 and HPeV6-550389 [12,23,24]. HPeV1-Harris and the HPeV2-751312 strains were provided by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands, and passaged to obtain a sufficient virus stock. The HPeV3 to 6 were isolated from stool passaged two

to three times to obtain sufficient virus stocks. HPeV1, 2, 4, and 5 were cultured in the HT29 cell line, HPeV3 in the Vero cell line and HPeV6 in the RD99 cell line and the virus working stocks were stored in aliquots at -80°C. The virus concentration was determined by the median tissue culture infective dose (TCID<sub>50</sub>) and calculated by the Reed and Muench method [25].

### Antibodies

The anti-HPeV (aHPeV) Abs against HPeV1 (Harris) and 2 (Williamson) were obtained from a rabbit Ab pool prepared at the RIVM. The aHPeV3 (A308-99) Ab was a kind gift from Dr. Shimizu, National Institute of Infectious Diseases, Tokyo, Japan, prepared as pooled guinea pig serum [10]. The aHPeV4 (S2592) and aHPeV5 (S2663) Abs were a kind gift from Dr. Schnurr, Viral and Rickettsial Disease Laboratory, California Department of Health Services, Berkeley USA, prepared as Armenian Hamster pooled serum [26].

### Virus replication curves

Monolayers of the different cell lines were cultured in 24 wells plates (Cellstar) with 1 ml medium and incubated at 37°C, 5% CO<sub>2</sub>. At day 0 the cell lines with a confluence of ~80% were infected with HPeV at a multiplicity of infection (MOI) of 0.001 in a volume of 200 µl culture medium for two hours, after which the non absorbed virus was removed and replaced with 1 ml maintenance medium (EMEM 2%, DMEM 2%) and incubated for 10 days. The low MOI is chosen to elucidate the entire process of the infection cycle of HPeV in cell culture. Twenty µl culture supernatant was removed for RNA extraction and quantitative RT-PCR detection at days 0, 1, 3, 7 and 10. The supernatant was extracted by automatic extraction using the total nucleic acid isolation kit with the MagnaPure LC instrument® (Roche Diagnostics). The RNA was eluted in 50 µl elution buffer and reverse transcribed as described previously [12]. Five µl of cDNA was used for real-time PCR using the LC480 (Roche Diagnostics) [12]. The virus copies per PCR were calculated with a standard curve as described previously [13]. The virus replication was normalized to the number of virus copies per PCR on day 0 (input virus). At day 10, supernatants were genotyped to confirm the input virus strain by VP1 genotyping as described before [13]. All replication experiments were first optimized with different MOI and all experiments were done in two-fold.

### Neutralization assay

Abs were mixed with the different HPeV1 to 6 virus suspension containing 100 TCID<sub>50</sub>/50 $\mu$ l. Ab dilutions of aHPeV1, aHPeV2, aHPeV3, aHPeV4 and 5 (1:100) were used for end-point neutralization of 100TCID<sub>50</sub> HPeV1 to 6. Mixtures were incubated at 37°C for 1hr, and were used to inoculate HT29 cells (HPeV1, 2, 4, 5, and 6) and Vero cells (HPeV3) on a 96-wells plate (200  $\mu$ l). Virus, cell and Ab controls were included as positive and negative control. The cells were examined for the appearance of CPE every 24hrs for 3 and 7 days and amount of viral copies were defined by real time PCR.

### Competing interests

The authors declare that they have no competing interests.

### Authors contributions

BW, KB, KW contributed to the study design. BW, SM, SJ contributed to acquisition of data, and analysis and interpretation of the data. BW, KB, KW have been involved in preparing the manuscript. All authors read and approved the final manuscript.

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# 3.

## **Specific Cell Tropism and Neutralization of Human Parechovirus Types 1 and 3: Implications for Pathogenesis and Therapy Development**

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## Abstract

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

## Introduction

Human parechoviruses (HPeVs) are small, non-enveloped, single-stranded positive-sense RNA viruses within the genus *Parechovirus* in the family *Picornaviridae*. HPeV1 and HPeV2 were originally described as echoviruses 22 and 23 within the genus *Enterovirus*, based on their similar cytopathic effect (CPE) on monkey kidney cells (Wigand & Sabin, 1961). However, sequence analysis showed that they were genetically distinct from other enteroviruses (EVs) and they were reclassified as HPeV1 and HPeV2 in the genus *Parechovirus*. Nowadays, 16 genotypes are known but only HPeV1 to HPeV6 were shown to grow in cell culture (Al-Sunaidi *et al.*, 2007; Benschop *et al.*, 2006b; Drexler *et al.*, 2009; Ito *et al.*, 2004; Li *et al.*, 2009; Watanabe *et al.*, 2007). While EVs are found in individuals of all ages, HPeVs mainly infect children under 5 years old and are usually associated with mild gastrointestinal and respiratory symptoms. An exception is HPeV3, which was first isolated in Japan from a 1-year-old patient with transient paralysis (Ito *et al.*, 2004). HPeV1, HPeV2 and HPeV4–6 are all mainly associated with milder symptoms; HPeV3 infections cause central nervous system (CNS) infections and neonatal sepsis (Benschop *et al.*, 2006a; Boivin *et al.*, 2005; Harvala *et al.*, 2009; Pajkrt *et al.*, 2009; Verboon-Maciolek *et al.*, 2008). The differences in clinical manifestations between HPeV types may be explained by differences in biological characteristics, such as cell tropism. HPeV3 is more difficult to grow in cell culture than HPeV1 (Benschop *et al.*, 2010a; Boivin *et al.*, 2005; Watanabe *et al.*, 2007). HPeV3 lacks the arginine-glycine-aspartic acid (RGD) motif located in the C terminus of the capsid protein VP1, which has been shown to be essential for HPeV1 receptor binding and entry (Boonyakiat *et al.*, 2001; Joki-Korpela *et al.*, 2001; Triantafilou *et al.*, 2000). The differences in *in vitro* growth characteristics (Benschop *et al.*, 2010a; Watanabe *et al.*, 2007) and the lack of the RGD motif imply use of a different cellular receptor by HPeV3 from that used by HPeV1, and a potentially different cell tropism. In addition, we showed that frequent recombination occurs between the HPeV genotypes except for HPeV3 (Benschop *et al.*, 2008c). This may also imply a different cell tropism of HPeV3, as infection of different cell types *in vivo* might reduce the opportunity for recombination to occur. A relationship between clinical manifestations and growth characteristics *in vitro* has previously been suggested for EV71. Virus growth was monitored by measuring viral RNA in cell-culture supernatant, showing a fivefold increase of viral RNA 48 h post-infection (p.i.) of EV71 isolated from a patient with encephalitis on the neural cell line SK-N-SH (Wen *et al.*, 2003). A second study showed that the encephalitis EV71 strain exhibited better growth on PBMCs and astrocytes than the EV71 strain from a patient without CNS symptoms (Kung *et al.*, 2007).

In addition to differences in clinical symptoms, children infected with HPeV3 are significantly younger (<2 months) than children infected with HPeV1 (>6 months) (Benschop *et al.*, 2006a; Wolthers *et al.*, 2008). This might suggest a lack of protection by maternal neutralizing antibodies (nAbs) in newborns against HPeV3. For HPeV3, the seroprevalence among women of child-bearing age in Japan is 68% (Ito *et al.*, 2004), which is lower than the seroprevalence in Finland for HPeV1 in adults (99 %) (Joki-Korpela & Hyypia, 1998; Tauriainen *et al.*, 2007). For EVs, it has been shown that lack of maternal antibodies is a risk factor for severe disease in infants (Abzug *et al.*, 1995). In neonates with severe EV infection, intravenous immunoglobulins (IVIG) are used as treatment. Currently this is the only available treatment against severe EV infections, although evidence on its efficacy is lacking (Wildenbeest *et al.*, 2010).

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

## Results

### Replication kinetics of HPeV1 and HPeV3 patient strains

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

Six HPeV1 strains and seven HPeV3 strains isolated from 13 stool samples were selected according to the clinical manifestations and severity of disease documented for the patients (Table 1) and were cultured with the same input per cell culture (m.o.i. 0.001). Of the HPeV1-infected children, patients P1-1 to P1-4 showed mild gastrointestinal and/or respiratory symptoms; P1-5 in addition had sepsis-like illness (SLI) and P1-6 suffered from SLI with CNS involvement (Benschop *et al.*, 2006a; Wolthers *et al.*, 2008). In the HPeV3 group, P3-1 and P3-2 had mild disease without SLI or CNS symptoms, P3-3 had SLI without CNS involvement, P3-4 had CNS infection without SLI, and P3-5, P3-6 and P3-7 had SLI and CNS involvement.

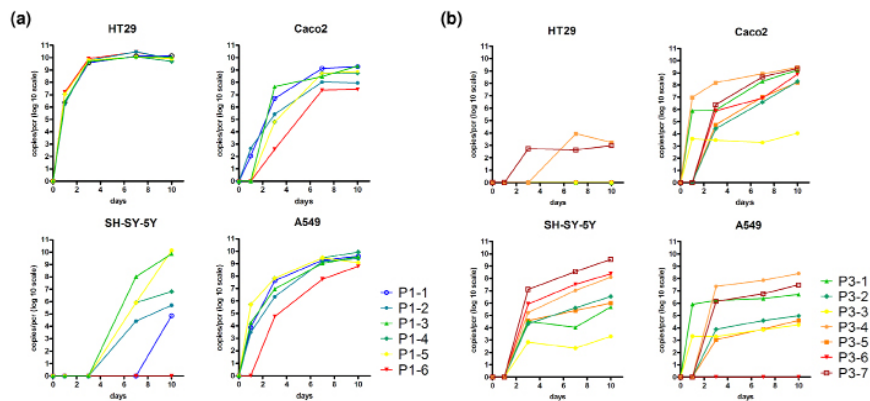
All HPeV1 and HPeV3 strains were able to replicate on the positive-control cell lines Vero and BGM (data not shown). P1-1 to P1-6 strains all showed remarkably similar and highly efficient replication kinetics on the HT29 cell line, with high levels of replication reaching  $>10^8$  copies per PCR at day 10 p.i. (Fig. 1a). While replication on the HT29 cell line was absent for five HPeV3 strains (P3-1, P3-2, P3-3, P3-5 and P3-6), we did observe low-level replication ( $<10^4$  copies per PCR) for P3-4 and P3-7, both strains from patients with CNS symptoms (Table 1; Fig. 1b). Remarkably, all HPeV3 strains were able to replicate on the other gastrointestinal cell line, Caco-2, with the same high titres as shown for HPeV1, with the exception of strain P3-3. Replication of both HPeV1 and HPeV3 strains was supported by the A549 and HEL cell lines, with the exceptions of strain P3-6 (A549) and strain P3-5 (HEL). The HPeV3 strains showed slower replication kinetics, reaching lower viral titres than HPeV1 strains on the A549 cell line (Fig. 1a, b), while all HPeV strains showed slow growth on the HEL cell line (data not shown). HPeV1 strains P1-1 to P1-5, isolated from the stools from patients with no signs of CNS involvement, did show replication on the neural cell line SH-SY-5Y, albeit with slow kinetics, starting replication at day 3 p.i. P1-6, isolated from the stool from a patient with CNS symptoms, was not able to replicate on the SH-SY-5Y cell line. In contrast, rapid replication on SH-SY-5Y cell line was observed for all HPeV3 strains. P3-4, P3-6 and P3-7, isolated from the stools from patients with CNS symptoms, showed up to a threefold higher viral RNA titre at day 10 p.i., compared with P3-1, P3-2 and P3-3 that were isolated from the stools from patients without CNS symptoms; however, P3-5 did not reach these higher titres, despite being isolated from a patient with CNS involvement (Table 1; Fig. 1b).

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

GI, Gastrointestinal symptoms; Resp, respiratory symptoms; SLI, sepsis-like illness; CNS, central nervous system symptoms.

Patient*	Age (months)	Symptoms			
		GI	Resp	SLI	CNS
P1-1	4,7	+	-	-	-
P1-2	9,9	+	-	-	-
P1-3	6	-	+	-	-
P1-4	5,6	+	+	-	-
P1-5	4,8	+	+	+	-
P1-6	0,7	-	-	+	+
P3-1	0,9	+	-	-	-
P3-2	10,6	+	+	-	-
P3-3	0,2	-	-	+	-
P3-4	10,8	+	-	-	+
P3-5	1,0	-	-	+	+
P3-6	0,2	+	+	+	+
P3-7	1,7	+	+	+	+

\*Numbers starting P1 and P3 indicate HPeV1- and HPeV3 infected children respectively



**Figure 1.** Replication kinetics of HPeV1 and HPeV3 patient strains. The HPeV strain numbers correspond with the patient numbers in Table I. (a) Replication kinetics of HPeV1 strains. P1-1, P1-2, P1-3, and P1-4 were isolates from patients with mild gastrointestinal and respiratory symptoms (green/blue colors), P1-5 from a patient with SLI (yellow), and P1-6 from a patient with SLI and CNS symptoms (red). (b) Growth kinetics of HPeV3 strains. P3-1 and P3-2 were isolated from patients displaying only mild symptoms (green/blue colors), P3-3 was isolated from a patient with SLI (yellow), P3-4 was isolated from a patient with CNS symptoms and P3-5, P3-6 and P3-7 were isolates from patients with both SLI and CNS symptoms (orange/red colors). Cells were infected with HPeVs at a MOI 0.001 and viral RNA was detected in the supernatant with RT-PCR. The 10log virus copies were calculated with a standard curve, and the input virus copies per PCR at time point 0 were subtracted.

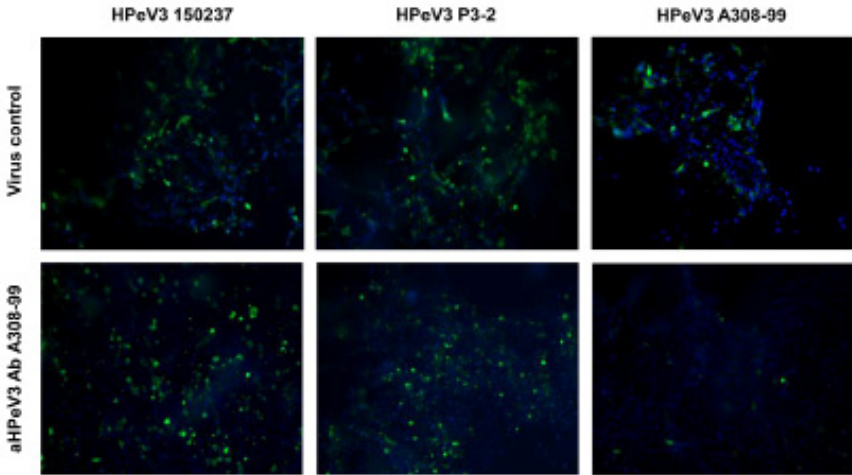
### Neutralization capacity

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

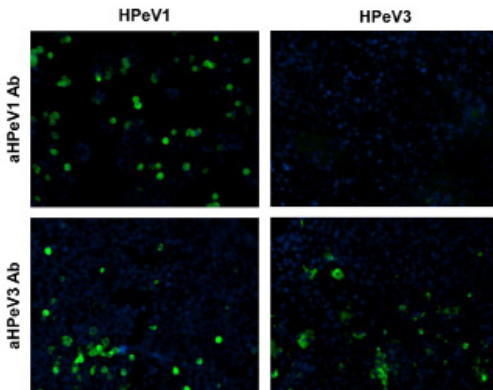
To test the neutralization capacity of IVIG against HPeVs, six different IVIG batches from 2005, 2008, 2009 and 2010 were used for neutralization. High neutralizing titres against HPeV1 were found (>1:1280), while neutralization titres against HPeV3-150237 and HPeV3 A308-99 were very low (<1 : 20 and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

To further investigate neutralization of HPeV3, sera from two different donors, in whom an HPeV3 infection had been detected by PCR in a stool sample from the past year, were tested for neutralization by CPE as well as PCR read-out (Fig. 4). High nAb titres against HPeV1 were found in both donors. Sera of donors 1 and 2 showed nAb titres of 1 : 256 and 1 : 4096, respectively, at time point 1, indicating previous infection with HPeV1. After 1 year, the sera of donors 1 and 2 had nAb titres of 1:512 and >1:4096, respectively, indicating that nAbs can still be strong at least 1 year after infection. An 8 log<sub>10</sub> difference was measured in viral copies by PCR between non-neutralized and neutralized virus infection (Fig. 4a), and virus growth could only be visualized by IFA starting at the indicated serum antibody dilutions (Fig. 4b). However, for HPeV3 these high nAb titres could not be found after infection. At time point 2, 1 year after infection, incomplete inhibition of virus growth could be shown by an antibody titre of 8, while no growth inhibition could be found by antibody titres of 32 and upwards (Fig. 4b). Virus replication measured by PCR decreased with a maximum of 2 log<sub>10</sub> only at the very low nAb titres (<1 : 16) (Fig. 4a). Therefore, in two adult donors 1 year after HPeV3 infection, partial neutralization can be found only at very low serum antibody

titres ( $<1 : 16$ ), which is in contrast to the high serum nAb titres against HPeV1 found in these two adult donors without apparent recent HPeV1 infection.



**Figure 2.** Neutralization ability of aHPeV3 Ab. Immuno- fluorescence assay for detection of HPeV3- 150237, A308-99 and P3-2 after HPeV3- A308 99 Ab neutralization. A standard neutralization assay was performed, the HPeV3 strains were pre-incubated with the polyclonal A308- 99 Ab and used for infection in Vero cell. Cells were stained at day 7 with polyclonal Abs against HPeV3, and secondary FITC labelled goat IgG Abs (green). The nuclei of the cells is stained with DAPI (blue).



**Figure 3.** Immuno- fluorescence HPeV1 and HPeV3 Ab binding. Immuno- fluorescence assay for detection of HPeV1 infection in HT29 cells and HPeV3 infection in Vero cells. Cells were stained when a CPE of 2+ was reached with polyclonal Abs against HPeV1 and 3, and secondary FITC labelled goat IgG Abs (green). The nucleus of the cells is stained with DAPI (blue).

**Table 2.** Neutralization of HPeV1 to 6 by polyclonal Abs aHPeV1 and aHPeV3, measured by CPE at day 7 post infection.

-, No CPE; 1+, 0–25% of infected cells showing CPE; 2+, 25–50% CPE; 3+, 50–75% CPE; 4+, 75–100% CPE.

	aHPeV1-Ab <sup>‡</sup>	aHPeV3 Ab <sup>§</sup>	No Ab
HPeV1- Harris <sup>*</sup>	-	4+	4+
HPeV2- 751312 <sup>*</sup>	4+	4+	4+
HPeV3- 150237 <sup>*</sup>	3+	3+	3+
HPeV4- 251176 <sup>*</sup>	4+	4+	4+
HPeV5- 552322 <sup>*</sup>	4+	4+	4+
HPeV6- 550389 <sup>†</sup>	4+	4+	4+

<sup>\*</sup>Ab dilution for HPeV1 was 1:10.

<sup>§</sup>Ab dilution for HPeV3 was 1:2.

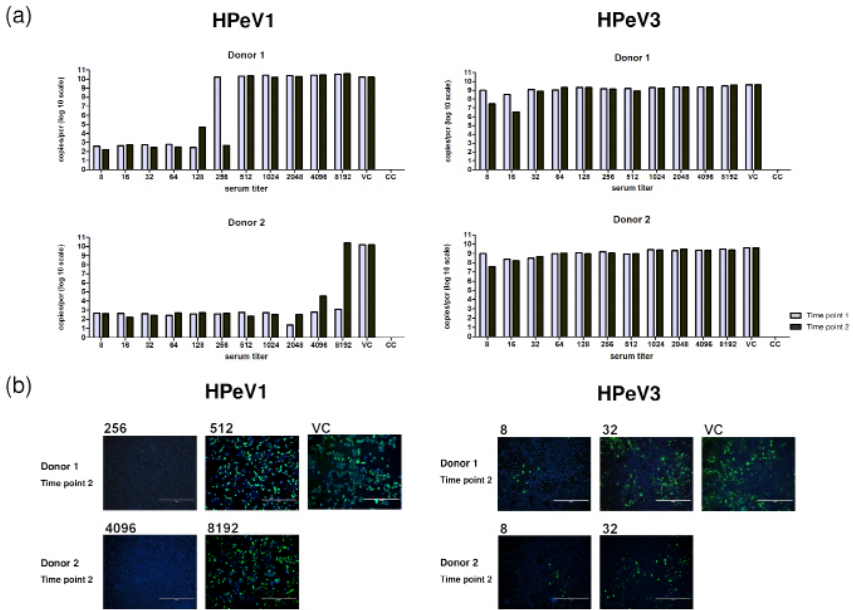
<sup>\*</sup> 100TCID<sub>50</sub> infection on HT29 (HPeV1, HPeV2, HPeV4, HPeV5) and Vero (HPeV3) cells.

<sup>†</sup> 1000TCID<sub>50</sub> infection of HT29

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

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





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

## Discussion

Differences in disease manifestations and severity between EV types can be related to differences in cell tropism and antibody neutralization capability (Abzug *et al.*, 1995; Kung *et al.*, 2007; Wen *et al.*, 2003). In this study, we used PCR to show differences in replication kinetics of HPeV1 and HPeV3 strains from patients with known clinical symptoms. The HPeV3 strains showed better replication kinetics on the neural cell line SH-SY-5Y. Virus strains isolated from three of four patients with CNS involvement showed better replication on the neural cell line SH-SY-5Y, reaching up to threefold higher viral titres, than viruses from patients without CNS involvement. For HPeV1, a correlation between *in vitro* replication dynamics and disease severity could not be found. Of note, these strains have all been isolated from stool samples and therefore do not necessarily represent the key features needed for neural tropism. Interestingly, HPeV3 was able to replicate well on the Caco-2 cell line, while only two strains could replicate (inefficiently) on the HT29 cell line. Caco-2 cells are known for their possibility to differentiate to a more fetal-like phenotype rather than adult ileal enterocytes, resulting in different receptor expression (Engle *et al.*, 1998; Levy *et al.*, 1998). This phenotype apparently is more suitable for HPeV3 replication than the phenotype of the other gastrointestinal cell line, HT29. Given the lack of the RGD motif of HPeV3, it is likely that HPeV3 uses a different receptor from HPeV1. However, more research is needed to elucidate the mechanisms of differential cell tropism. The cell lines used in our study are continuously growing cell lines obtained from tumours that do not necessarily represent the tissue from which they originated. Receptor expression and post-entry translational mechanisms might differ from their *in vivo* counterparts, which is a limitation for pathogenesis studies. To get better representation of the *in vivo* replication, primary cell systems need to be established.

For picornaviruses, the interaction of the antibody with the RGD motif has shown to be important for virus neutralization (Verdaguer *et al.*, 1995). For HPeV1 it has been shown that, next to VP1, VP0 also contains important antigenic sites (Alho *et al.*, 2003; Joki-Korpela *et al.*, 2000). We showed that our HPeV3 strains could not be neutralized by the polyclonal specific antibody elicited against the Japanese HPeV3 prototype, while the antibody did neutralize the Japanese strain, albeit at a lower titre than reported previously. This could indicate that the antibody has partly lost its potency. Interestingly, the Japanese HPeV3 strain A308-99 is genetically very similar to our strains (97–100 %) (Benschop *et al.*, 2006b, 2008c, 2010b). Subsequently, IVIG was tested for HPeV nAb titres. IVIG represents IgGs from >1000 donors and is sometimes given to reduce the disease burden from severe EV infection (Wildenbeest *et al.*, 2010). IVIG manufactured between 2005 and 2010 by our local blood bank contained high nAb titres against HPeV1, but could hardly neutralize

HPeV3 strains, showing that IVIG is not suitable as an antiviral treatment against HPeV3. One could argue that HPeV3 is a newly circulating type and thus there is no high antibody immunity against HPeV3 within the population. However, HPeV3 has been shown to be the second most circulating type since the beginning of the twenty-first century (Benschop *et al.*, 2010a; van der Sanden *et al.*, 2008) and has been present since 1994 (Benschop *et al.*, 2010b). Finally we observe very low nAb titres in serum of two HPeV3-infected donors, while high nAb titres against HPeV1 could be detected. A limitation to the study is that neutralization experiments were performed on different cell lines, since optimal HPeV1 and HPeV3 replication is found on different cell lines. From our data we conclude that HPeV3 is difficult to neutralize *in vitro*; whether this is the case for the *in vivo* situation needs to be elucidated further. Based on our observations that HPeV3 is associated with severe disease in young infants, we previously suggested a lack of maternal protection against HPeV3 infection (Benschop *et al.*, 2006a; Harvala *et al.*, 2010; Wildenbeest *et al.*, 2010). Based on the low nAb titres found against HPeV3, despite efficient antibody binding, we now hypothesize that antibody protection against HPeV3 might fail. Therefore, other host and/or viral factors must be involved in susceptibility too, or in protection against infection, and these factors will determine the clinical course of HPeV3 infection in young infants. It could be that the virus structure does not permit antibodies to reach the neutralizing epitope. New mono- and polyclonal antibodies are needed to elucidate the mechanisms of neutralization of HPeV3 and get more insight into potential antiviral drug targets.

In summary, a relationship between replication efficacy on neural cell lines and CNS infection for HPeV3 was found, while this could not be shown for HPeV1. In addition, HPeV3 was difficult to neutralize with its specific antibody, serum antibodies and IVIG, while efficient neutralization with high titres was found for HPeV1 with aHPeV1 antibody, serum and IVIG. Our study provides new insights into the cell tropism and antibody protection of HPeVs, which are important factors for disease severity. These new insights address the need for antivirals against HPeVs and are essential in the development of treatment strategies and development of these antivirals.

## Methods

**Cell lines.** For virus culture, the following cell lines were used: human colon carcinoma (HT29), human colon adenocarcinoma (Caco-2), human lung carcinoma (A549; kindly provided by the University Medical Center, Leiden), human embryonic lung cells (HEL), rhabdomyosarcoma (RD99; kindly provided by the Dutch National Institute for Public Health and the Environment), African green monkey kidney (Vero), buffalo green monkey kidney (BGM), Rhesus monkey kidney (LLCMK2; kindly provided by the Municipal

Health Services, Rotterdam) and human neuroblastoma (SH-SY-5Y; kindly provided by Dr Tauriainen, University of Tampere, Finland). The cells were maintained in Eagle's minimum essential medium (EMEM; Lonza) supplemented with L-glutamic acid (0.2X; Gibco), non-essential amino acids (1X; Gibco), streptomycin (0.1 µg/ ml-1 Sigma) for 1 week before being passaged. For HT29, A549, HEL, RD99, Vero, LLCMK2 and BGM cell lines, the medium was supplemented with 8 % heat-inactivated FCS (Sigma) and, for the Caco-2 cell line, with 20 % heat-inactivated FCS. The human neuroblastoma cell lines were cultured in Dulbecco's modified Eagle medium (DMEM; Gibco) and supplemented with heat-inactivated 10% FCS, L-glutamic acid (0.2X), non-essential amino acids (1X), streptomycin (0.1 µg/ ml-1) and ampicillin (0.1 µg/ ml-1). Fresh medium containing 2% FCS was added to the cells 3-4 days after passaging.

**Virus strains and cultivation.** The following HPeV strains were used as laboratory prototypes: HPeV1A Harris, HPeV2-751312, HPeV3-150237, HPeV4-251176, HPeV5-552322 and HPeV6-550389 (Benschop *et al.*, 2006a, 2008b, 2010a). In addition, a selection of HPeV1 and HPeV3 strains isolated from patients with well-documented clinical syndromes (Table 1) was made. The Japanese patient strain A308-99 was a kind gift from Dr Shimizu, National Institute of Infectious Diseases, Tokyo, Japan (Ito *et al.*, 2004). The viruses were passaged two or three times to obtain sufficient virus stocks. HPeV1, HPeV2, HPeV4 and HPeV5 were cultured in the HT29 cell line, HPeV3 in the Vero cell line and HPeV6 in the RD99 cell line and the virus working stocks were stored in aliquots at -80°C. The virus concentration and calculated by the method of Reed & Muench (1938).

**Antibodies.** The anti-HPeV (aHPeV) antibody against HPeV1 (Harris) was obtained from a rabbit antibody pool prepared at the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. The aHPeV3 (A308-99) antibody was a kind gift from Dr Shimizu, National Institute of Infectious Diseases, Tokyo, Japan, prepared as pooled guinea pig serum (Ito *et al.*, 2004).

**Virus replication curves.** Monolayers of the different cell lines were cultured in 24-well plates (Cellstar) with 1 ml medium and incubated at 37 °C, 5% cell lines were infected with HPeV isolates at an m.o.i. of 0.001 in a volume of 200 µl culture medium for 2 h, after which the non-absorbed virus was removed and replaced with 1 ml maintenance medium (2 % EMEM, 2% DMEM) and incubated for 10 days. The low m.o.i. is chosen to elucidate the entire process of the infection cycle of HPeV in cell culture. At days 0, 1, 3, 7 and 10, culture supernatant was removed for RNA extraction and quantitative RT-PCR detection. The supernatant (20µl) was extracted by automatic extraction using a total nucleic acid isolation kit with the MagnaPure LC instrument (Roche Diagnostics). The RNA was eluted in 50

ml elution buffer and reverse-transcribed as described previously (Benschop *et al.*, 2010a). cDNA (5 µl) was used for real-time PCR using a LightCycler 480 (Roche Diagnostics) (Benschop *et al.*, 2010a). The virus copies per PCR were calculated using a standard curve as described previously (Benschop *et al.*, 2008a). Virus replication was normalized to the number of virus copies per PCR (input virus, day 0). At day 10, supernatants were genotyped to confirm the input virus strain by VP1 genotyping as described before (Benschop *et al.*, 2008a). All experiments were performed twice with reproducible results.

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

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

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# 4.

## **Efficient replication of the arginine-glycine-aspartic acid (RGD) lacking Human Parechoviruses in 3D Human Airway Epithelial Cell models**

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## Abstract

Human parechovirus (HPeV) infections are mainly found in children, associated with a broad range of symptoms, including respiratory symptoms. The respiratory epithelium is the initial tissue exposed to HPeVs since transmission of HPeVs is supposed to be oral-fecal, suggesting a role for respiratory epithelium in HPeV entry and replication. Difficulties have been shown in detection of specific HPeV types in cell culture, most pronounced for HPeV3 and the more recently detected HPeV7-16, which have never been successfully propagated in cell culture. HPeV3 and the newer genotypes HPeV7-16 lack the arginine- glycine- aspartic acid (RGD) motif in the VP1 capsid region. This region is essential for HPeV1 receptor binding and entry.

In this study we used primary well-differentiated human airway epithelium (HAE) cell cultures from different origin: an in house bronchial HAE as well as commercial bronchial and nasal HAE cells MucilAir™ (Epithelix). We showed efficient replication of HPeV3 and to a lesser extent of HPeV1 in contrast to what has been shown before using standard cell culture systems. The new RGD-lacking genotypes HPeV9 and HPeV14 and a recombinant HPeV3 strain, which were not able to replicate on standard cell cultures, could be propagated on HAE. Therefore, the HAE cell culture system provides a new tool for further studies on tropism and pathogenesis of RGD-less HPeV types, which could lead to the further characterization of receptors for the RGD-lacking HPeVs and other human picornaviruses.

## Introduction

The human parechoviruses (HPeVs) belong to the family *Picornaviridae*, a large and diverse group of positive sense RNA single stranded viruses (1). HPeVs are widespread, causing disease mainly in young children, with clinical symptoms ranging from mild respiratory and gastrointestinal symptoms to severe neonatal sepsis and infections of the central nervous system (CNS) (2). The genus *Parechovirus* contains 16 HPeV genotypes based on sequencing of the VP1 region (3). The VP1 region encodes for one of the three capsid proteins VP0, VP3 and VP1. The C-terminus of the VP1 capsid protein of HPeV1, 2, 4, 5 and 6 contain the arginine- glycine- aspartic acid (RGD) motif, which has been shown to be essential for HPeV1 receptor binding and entry (4–6). HPeV3 and the more recently identified types HPeV7-16 lack this RGD motif suggesting an alternative receptor or entry route for these viruses. Indeed, several studies showed difficulties to detect HPeV3 in cell cultures regularly used for HPeVs because only a low induction of cytopathic effect (CPE) could be scored (7, 8). Similarly, the newer HPeV7-16 were all detected by PCR in patient derived samples and could not be cultured in standard cell lines (9–11). For HPeV3 we showed that when virus replication in cell culture was monitored by RT-PCR instead of CPE, most cell lines supported replication of HPeV3 (12, 13). Still several HPeV strains, mostly lacking the RGD motif, were unable to replicate in our standard cell lines. We discovered an HPeV1 strain (HPeV1-652281) that was closely related to the prototype HPeV1-Harris strain but lacked the RGD motif, despite its essential role for HPeV1 in utilising the  $\alpha v\beta 3$ ,  $\alpha v\beta 6$  integrins as receptors (5, 6, 14, 15). Furthermore, we discovered an RGD-less HPeV5 (HPeV5-652444). Both strains showed a specific sequence; Isoleucine- Serine- Asparagine (ISN)/ Threonine- Serine- Asparagine (TSN) at the position of the RGD motif where HPeV3 has a deletion (9). Previously we described an interesting HPeV3 strain that could not be cultured (16). This HPeV3-651689 is the only HPeV3 strain that does not exclusively cluster in phylogenetic trees with HPeV3 in both structural as non-structural genomic regions. In the non-structural regions HPeV3-651689 mainly clustered with HPeV7 PAK5045 as shown by phylogenetic analysis of whole genomes (11, 16). Of the newer HPeV genotypes, HPeV14 (HPeV14-451564) was first discovered in our laboratory by PCR screening in faeces (9), while HPeV9, first described by Oberste *et al.*, (Oberste *et al.*, unpub., <http://www.picornaviridae.com>), was found once in our samples in 2010 (HPeV9-1051908). An RGD-containing strain that could not be cultured was HPeV2-950912, showing distinct features from the HPeV2-Williamson strain; HPeV2 is hardly detected in the Netherlands (9, 17). HPeV strains lacking the RGD motif show differences in *in vitro* growth characteristics compared to RGD containing HPeVs such as HPeV1 (13) thereby implying use of an (unknown) different cellular receptor and thus a potentially different cell tropism.

The respiratory epithelium is the initial tissue exposed to HPeVs since transmission of HPeVs is supposed to be oral-fecal, thus suggesting a role for respiratory epithelium in HPeV entry and replication. Recently, primary human airway epithelial (HAE) culture systems have become available, closely mimicking the *in vivo* human respiratory environment (18–20). Several viruses that could not be propagated in standard cell culture indeed have successfully been cultured in these HAE culture system, such as rhinovirus C, a picornavirus species belonging to the Enterovirus genus (19, 21, 22). Therefore we used the HAE cell culture as a primary culture system to study replication of RDG-less HPeV strains.

## Materials and Methods

### Virus strains

The following HPeV strains were used as prototypes: HPeV1A Harris, provided by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands, HPeV2-2008, HPeV4-251176, HPeV5-552322 and HPeV6-550389 and passaged to obtain a sufficient virus stock; HPeV3-150237 (23) isolated from stool and passaged two to three times to obtain sufficient virus stocks. HPeV1, 2, 4, 5 and 6 were cultured on HT29 cells, HPeV3 on Vero cells; both cell lines were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with L-glutamic acid (0.2X), non-essential amino acid (1X), streptomycin (0.1 µg/ml) and ampicillin (0.1 µg/ml). The virus working stocks were stored in aliquots at -80°C. The virus concentration was determined in standard cell culture by the median tissue culture infective dose (TCID<sub>50</sub>) and calculated by the Reed and Muench method (24). Stool samples containing a selection of different HPeVs which could not be cultured in standard cell culture, HPeV1-652281, HPeV2-20950912, HPeV3-651689 (16), HPeV5-652444 (9), HPeV9-1051908, HPeV14-451564 (9) were used for further culture on HAE.

### HAE cell culture.

Normal primary human bronchial epithelial cells (HBEpC) were derived as described previously (18, 21), the HBEpC were maintained at 37°C in a 5% CO<sub>2</sub> incubator for one or two serial passages as a monolayer in bronchial epithelial cell serum-free growth medium (BEGM) (20), medium was refreshed at 2- or 3-day intervals. At 75% confluence, cells were dissociated with 2 ml of TrypLE Express enzyme (Invitrogen). HTEpC were diluted in air-liquid interface (ALI) medium (20). A total of  $2 \times 10^5$  HTEpC were seeded on 12-well or  $7 \times 10^4$  were seeded on 24-well ThinCerts with a 0.4-µm pore size (Greiner Bio-One). The inserts were pre-coated with type IV collagen (Sigma). Medium was renewed every 2 or 3 days, the apical medium was removed when reaching 100% confluence, creating an air

liquid interface. Medium from the basolateral compartment was renewed every 2 or 3 days and the apical surface was washed every week with Hanks balanced salt solution (HBBS) (invitrogen). The cultures were maintained for 30 days to let the cells differentiate into well-differentiated pseudostratified human airway epithelium.

For comparison of our in house bronchial HAE, bronchial and nasal epithelial cells from MucilAir™ (Epithelix) were used. MucilAir™ ready-to-use culture medium (Epithelix) from the basolateral compartment was renewed every 2 or 3 days.

### **HPeV infection of HAE**

The HAE cells were infected with the different HPeVs, 150 µl of 100 TCID<sub>50</sub>/50 µl virus stock in 12 well inserts or 50 µl of 100 TCID<sub>50</sub>/50 µl in 24 well inserts. In case of the clinical HPeV strains 100 µl of 0.22 µm filtered clinical specimen was used for inoculation of the nasal or bronchial 24 well inserts. The virus samples were added apically and after 3 hours incubation at 37°C in a 5% CO<sub>2</sub> incubator, the inoculum was removed and the cells were washed with HBSS for 10 min at 37°C, this was considered as timepoint 0. Samples were collected from the apical and the basolateral side every 24 hours post infection.

### **RNA isolation and detection of HPeV by real-time reverse transcription-PCR**

From all harvested apical wash and basolateral supernatant 25 µl was removed for RNA extraction and quantitative RT-PCR detection at 0, 24, 48 and 72 hours post infection. The supernatant was extracted by automatic extraction using the total nucleic acid isolation kit with the MagnaPure LC instrument® (Roche Diagnostics). The RNA was eluted in 50 µl elution buffer and reverse transcribed as described previously (7). Five µl of cDNA was used for real-time PCR using the LC480 (Roche Diagnostics). The virus copies per PCR were calculated with a standard curve as described previously (25).

### **Immuno- fluorescence assay**

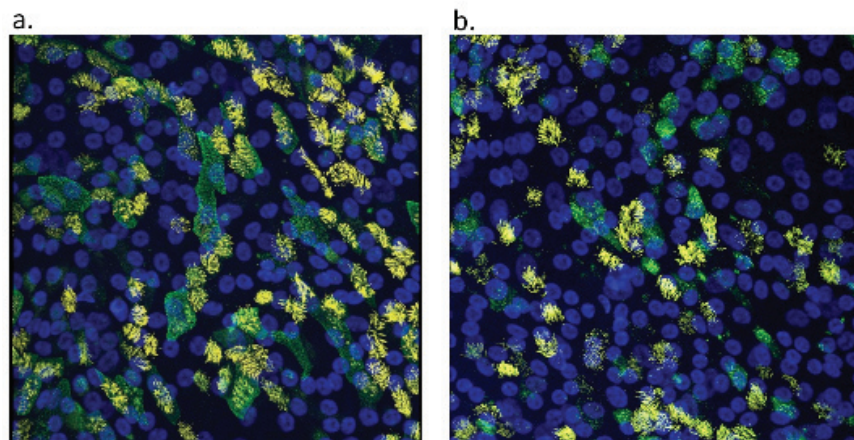
Polyclonal antibodies (Abs) used in fluorescence assays against HPeV1 and HPeV3 were obtained by immunization of rabbits (Harlan Laboratories). For detection of viral infection the cells were fixed apical and basolateral with 4% para-formaldehyde (PFA)/ PBS for 30 minutes. The PFA was removed and the cells were washed consecutively 3 times with PBS. To avoid unspecific binding cells were blocked with PBS supplemented with 50 mM NH<sub>4</sub>Cl, 0.1% saponin and 2% BSA (confocal staining buffer (CB)). After incubation with CB for 60 min apical and basolateral, the apical side is incubated for 1 hour at RT with the primary antiHPeV1 or antiHPeV3 polyclonal Abs and with anti-β-tubulin IV for staining the ciliated cells. Cells are washed 3 times (5') with CB and incubated with the secondary anti-rabbit secondary goat Abs - fluorescein isothiocyanate (FITC) labelled IgG

(Jackson Immuno Research), antimouse Cy5 labelled IgG and 0.5  $\mu\text{g}/\text{ml}$  4',6-diamidino-2-phenylindole (DAPI, Sigma) were added and incubated for 1hr 37°C. After washing, the membrane containing the cells was cut out and mounted on a glass slide. The cells were analysed on a Leica SP8 confocal microscope.

## Results

### Efficient HPeV3 replication in bronchial HAE cells

Well-differentiated human airway epithelial cells isolated from an adult donor were infected with the prototype HPeV strains HPeV1A Harris and HPeV3-150237 to determine replication on primary human cells. Replication was measured by determining the virus RNA load by real-time PCR on time samples from apical washes and the basolateral medium.

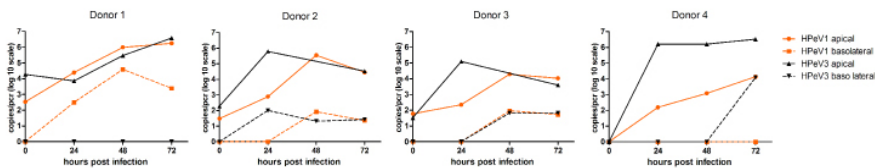


**Figure 2.** Confocal images of HPeV1 and HPeV3 infected HAE cells. Virus was detected 72 hours post infection with rabbit polyclonal antiHPeV1 or antiHPeV3 polyclonal Abs, visualized by anti-rabbit secondary goat Abs – FITC (green). Ciliated cells were visualized with anti- $\beta$ -tubulin IV, anti-mouse Cy5 labelled IgG (Yellow) and the nucleus was stained with DAPI (blue).

The infected HAE cultures did not show recognizable CPE, but viral RNA could be measured after 24 hrs. HAE cells from 5 different adult donors were infected to study reproducibility and donor variation. In 4 out of 5 donors, replication of HPeV1 and 3 could be detected (Fig. 1). In donor 5 no replication could be detected within 72 hrs post infection (data not shown). In contrast to what we have found previously using standard cell culture, replication

of HPeV3 on HAE was more efficient compared to HPeV1 in 3 out of 4 donors, (Fig. 1, donors 2, 3 and 4). Peak infection of HPeV3 was observed after 24 hr in donor 2, 3 and 4 (between  $10^5$  and  $10^6$  viral copies/PCR), whereas HPeV1 reached the highest titers after 48 or even 72 hr post infection. Next to comparison of HPeV1 and HPeV3 in HAE we infected the bronchial cells with HPeV2, 4, 5 and 6. HPeV2 replicated on HAE with maximum titers of  $10^6$  viral copies/PCR 72 hr post infection while replication of HPeV4, 5, and 6 was very low or even absent (n=1, data not shown).

To determine which type of cells from the well-differentiated epithelial culture are infected, viral antigen was identified in the HAE cells by immunofluorescence against HPeV1 and 3, while ciliated cells were distinguished from non-ciliated cells by  $\beta$ -tubulin IV staining. Viral antigen was detected in both ciliated and non-ciliated HAE cells infected with HPeV1 or HPeV3 (Fig 2).

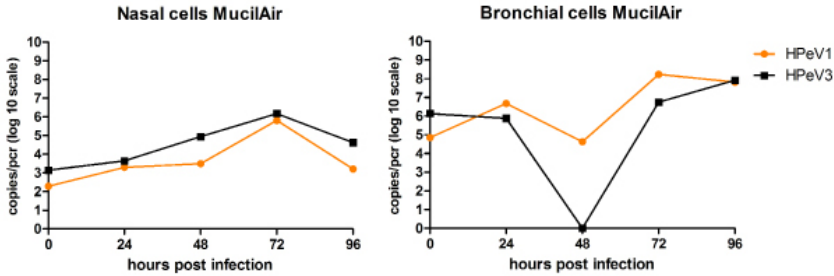


**Figure 1.** Replication of HPeV1 and HPeV3 on well-differentiated bronchial epithelial cells obtained from different donors. The viral replication was determined by measuring viral RNA in the apical wash and the basolateral medium at 24, 48 and 72 hours post infection. HPeV1: orange line- apical, dotted orange line- basolateral, HPeV3: black line- apical, dotted black line- basolateral..

### HPeVs replication in MucilAir™

To compare replication of HPeV1 and 3 in upper and lower respiratory tract cells, commercially available well-differentiated cells from nasal and bronchial origin (MucilAir™, Epithelix isolated from Caucasian elderly women), were infected with HPeV1 and 3. HPeV1 and HPeV3 showed similar replication patterns; in nasal cells replication could be measured after 24 hr post infection, but the highest titer was reached after 72 hr post infection ( $10^6$  copies/PCR). In bronchial cells even higher titers were reached, up to  $10^8$  copies/PCR. The replication curves were different from our in house HAE system (Figures 1 and 3). Notable is the dip in bronchial cells after 48 hr where no replication could be measured for HPeV3 and lower replication for HPeV1. This pattern could be reproduced in bronchial cells from two different donors.

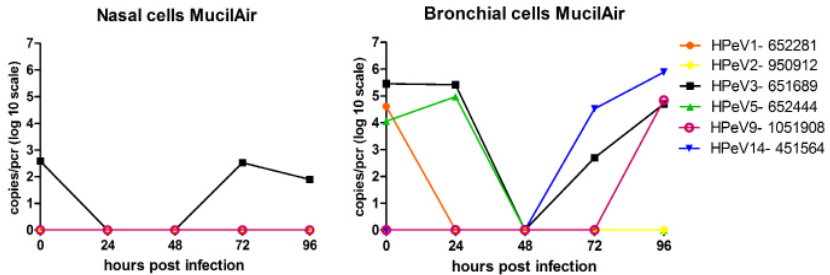




**Figure 3.** Replication of HPeV1 and HPeV3 on commercial nasal and bronchial epithelial cells (MucilAir™). The viral replication was determined by measuring viral RNA in the apical wash and the basolateral medium at 24, 48 and 72 hours post infection. HPeV1: orange line, HPeV3: black line.

### Propagation of RGD-less HPeVs in MucilAir™

We subsequently infected both nasal and bronchial (MucilAir™, Epithelix) cells with diluted and filtered stool samples containing the RGD-less HPeV1-652281, HPeV5-652444, HPeV3-651689, HPeV9-1051908 and HPeV14-451564 and the RGD containing HPeV2-950912. HPeV3-651689 replicated on both nasal and bronchial epithelial cells showing a dip around 48 hr post infection, and reaching high titers ( $10^5$  copies/PCR) in bronchial cells but not on nasal cells ( $10^2$  copies/PCR after 96 hr post infection) (Fig 4). HPeV14-451564 showed a similar pattern as HPeV3-651689 in bronchial cells, reaching even higher titers around  $10^6$  copies/PCR 96 hr post infection. HPeV9-1051908 was able to replicate on bronchial cells, but the first positive samples were obtained only after 72 hr post infection. The HPeV1, 2 and 5 strains could not be propagated from their original sample on MucilAir™.



**Figure 4.** Infection of HAE bronchial and nasal cells (MucilAir™) with different clinical isolates containing HPeV1-652281, HPeV5-652444, HPeV3-651689, HPeV2-20950912, HPeV9-1051908, HPeV14-451564. Viral replication was followed by detection of viral RNA 24, 48, 72 and 96 hours post infection in de apical wash.

## Discussion

In this study efficient replication of HPeV3, and to a lesser extent of HPeV1, was demonstrated using primary well-differentiated human airway epithelial cell cultures in contrast to what is previously shown in standard cell culture systems (7, 8, 26). In addition, with the MucilAir™ HAE system, we were able to propagate patient-derived HPeV9 and HPeV14, as well as the recombinant HPeV3-651689, none of which we could propagate in standard cell culture. Unfortunately, our HPeV1, HPeV5 (which lack the RGD motif) and the outlier HPeV2 strain were not able to replicate. This is most likely due to a low infectious virus present in the faecal samples.

The HAE system seems to be suitable for propagation of RGD-less HPeV types, with higher replication in the bronchial cells, indicating that an as yet unidentified RGD-independent receptor is expressed on these cells. For HPeV1 it has been shown that it utilizes  $\alpha v \beta 3$  and  $\alpha v \beta 6$  integrins as its receptor, and that the interaction with the RGD motif in VP1 is essential (5, 14, 15). For the other HPeVs, the receptor is unknown, but the RGD containing strains (HPeV2, 4, 5, and 6) are likely to use the same integrins. Previous studies showed that the fibronectin  $\alpha v \beta 3$  and  $\alpha v \beta 6$  receptors are only expressed at low or even undetectable levels in normal healthy airways *in vivo* and *in vitro* (27, 28). This could explain the less efficient replication of HPeV1 we observed compared to HPeV3, and the low or even absent replication of HPeV4, 5, and 6 in HAE. However, HPeV1 is still able to efficiently replicate in HAE, suggesting that an  $\alpha v \beta 3$  and  $\alpha v \beta 6$  integrin independent entry pathway might be used in healthy airways. Apparently, the unknown receptor for HPeV3 and the other RGD-less

HPeVs is expressed on HAE cells. Respiratory cells may reflect the initial port for entry of HPeV3 and even for a subsequent CNS infection. For EV71 it has been shown that infection of rhesus monkeys with EV71 via the respiratory tract resulted in more severe neurological lesions compared to the orally infected monkeys (29). The efficient infection of HPeV3 of human airway might therefore give a good portal to the CNS. HPeV7-16 have only recently been isolated and have not been detected frequently up to now, therefore only little is known of their clinical outcome and thus involvement in severe HPeV infections.

A relationship between clinical manifestations and *in vitro* replication characteristics of EV71, CAV9 and Echo9 has previously been suggested (30–37). However, this data is inconsistent; not all Echo 9 strains isolated from patients with paralysis were able to cause paralysis in mice, while some strains isolated from non-symptomatic children did cause paralysis in mice (38). From our previous study we were not able to draw a clear relationship between pathogenicity and replication in cell lines *in vitro*. HPeV3 strains could replicate efficiently in neural cells, in correlation with their *in vivo* CNS involvement. However, this correlation could not be shown for all HPeV3 strains and not for HPeV1 (12). All these studies used continuously growing cell lines obtained from (human) tumours or mice, which do not necessarily represent the tissue infected during human infection. Primary cell culture systems provide a much better alternative for pathogenicity studies, in which cells are used representing the tissue which is exposed to viral infection, and containing the receptors expressed in human tissues. During this study we saw variation in replication between different donors as well as between the systems used. This indicates the importance of testing multiple donors for primary cell culture systems as a tool for further research of cell tropism and the RGD- independent entry pathway.

In this study, cells derived from adult tissue were used showing a surprisingly efficient replication of HPeV3. HPeV3 infects mainly young children, adults with symptomatic HPeV3 infection have only been reported in Japan, showing symptoms of myalgia, muscular weakness, sore throat and orchiodynia (39, 40). Despite large screenings studies, only a few HPeV3 infected adults have been described elsewhere (13). From our study it can be concluded that HPeV3 can infect adult HAE cells, indicating that entry is not the bottleneck explaining few adult infections in Caucasians. However, to investigate the differences in HPeV3 infection of respiratory tissue between children and adults, more studies are needed including infection of tissue obtained from of children.

### Acknowledgement

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# 5.

## Human Parechovirus seroprevalence in Finland and the Netherlands

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## Abstract

**Background.** Human parechoviruses (HPeVs) are RNA viruses associated with mild gastrointestinal and respiratory infections in children, but have also been linked to neonatal sepsis and CNS infections in infants. While the prevalence of HPeVs is known mostly among hospitalized populations, the knowledge of HPeV seroprevalence in the general population is poor.

**Objectives.** The aim of this study was to identify and compare the HPeV1-6 seroprevalence in Finnish and Dutch populations.

**Study design.** A type specific microneutralization assay was set up for detecting neutralizing antibodies (nABs) against HPeV types 1-6. Altogether 616 serum samples from Finnish and Dutch population were analyzed for antibodies against HPeVs. The samples were collected from Finnish children aged 1, 5 or 10 years, Finnish adults, 0- to 5-year-old Dutch children, Dutch women of childbearing age and Dutch HIV-positive men.

**Results.** In both adult populations, seropositivity was high against HPeV1 (99% in Finnish and 92% in Dutch samples) and HPeV2 (86% and 95%). Against HPeV4, the seropositivity was similar (62% and 60%). In Dutch adults, nABs against HPeV5 and 6 (75% and 74%) were detected more often than in Finnish adults (35% and 57%, respectively). In contrast, seropositivity against HPeV3 was as low as 13% in the Finnish and 10% in the Dutch adults. The seroprevalence of all HPeV types increased with age.

**Conclusions.** The seroprevalence of HPeVs is high in Finnish and Dutch populations and HPeV type 2 and types 4-6 are significantly more prevalent compared to earlier reports. The seroprevalence of antibodies observed against HPeV3 was low.

## Background

Human parechoviruses (HPEVs) are commonly occurring viruses that circulate especially among young children. Together, with rodent-borne Ljungan viruses [1] these small positive stranded RNA viruses form the *Parechovirus* genus within the family of *Picornaviridae*. HPeV1 and HPeV2 were first described in the 1950s from children with diarrhoea and classified in the genus Enteroviruses as echovirus 22 and 23 [2] Based on sequence analysis they were, however, reclassified [3] To date, 16 distinct HPeV genotypes are identified, but only genotypes 1-6 grow in cell culture [4-11].

Globally, HPeVs exist in children [12-15] and they cause mainly mild gastrointestinal and respiratory infections but also more severe, central nervous system (CNS) related symptoms and neonatal sepsis. In infants, HPeV3 is a significant cause for encephalitis, meningitis and sepsis-like illness [14,16-18], while other HPeVs have no clear relation to serious infections [19,20].

HPeV1 is the most common type worldwide while the detection of other types is less frequent [12,13,15,20-23]. In European studies the next common types are HPeV3, HPeV4 and HPeV6 [12,14,20,22] while in Asia the types distribution differs including types 8, 10 and 11 [15, 23], which to date have not been detected in Europe. Seroepidemiologic data on HPeV is lacking. In a Japanese study, the seroprevalence for HPeV3, among women of childbearing age, was 68% [8], which is lower than the seroprevalence found for HPeV1 in adults (99%) [24,25]. The seroprevalence of HPeV1 is high, reaching 72%, in children by the age of 2 years [25]. Seroprevalence data are lacking for other types than HPeV1 and 3 and this would provide more information on virus circulation in the population and existing immunity, which are important factors in understanding epidemiology and outbreaks. Moreover, it would facilitate more precise comparison of HPeV epidemiology among regions.

## Objectives

The study objective was to determine the seroprevalence of human parechovirus types 1-6 in Finnish and Dutch populations. Secondly, this collaborative study concentrated on examining the seroprevalence in two different geographic areas and in children and adult subpopulations.

## Study design

### Study population

Serum samples from Finland were collected from children whom participated in the Finnish Diabetes Prediction and Prevention (DIPP) birth cohort study [26]. Children were enrolled in this study before the age of 3 months and serum samples were collected regularly and stored frozen at  $-70^{\circ}\text{C}$  until processed. A set of samples from children aged of 1, 5 and 10 years (144, 149 and 147 samples, respectively) participating the diabetes study were selected for analysis. A total of 440 samples from altogether 250 children were tested. For each age: 1, 5, and 10 years - 61 children provided all 3 samples, one for each age group, 68 children provided 2 samples and 121 children provided a single sample. Sera from Finnish adults were collected from medical students ( $n=72$ ; mean age: 24; range: 21-40) from the University of Tampere Medical School. The Ethics Committee of Tampere University Hospital granted approval for the study of Finnish samples. The students and parents of each child provided a written informed consent.

Dutch sera were taken from the serum bank of the Laboratory of Clinical Virology, Academic Medical Center, Amsterdam. These samples were collected from patients admitted for virus diagnostics and stored at  $-20^{\circ}\text{C}$ . Sera were picked by random selection from defined target groups: (1) children up to 5 years; (2) women of childbearing age with a high probability of being in contact with young children, and defined by women admitted to the obstetrics ward; and (3) HIV-infected men, defined by HIV positivity. From each target group, 40 samples were randomly selected by laboratory number and renumbered, making the samples completely anonymous while age, sex, and target group were documented. For further analysis, 37 sera could be retrieved from children (mean age: 32 months; range: 0-67), 39 women between the ages 16 and 40 (mean age 30 years), and 38 from HIV-positive men (mean age 39 years, range 19-60). Altogether 114 Dutch serum samples were analyzed (mean age: 34 years; range 16-60). The use of patient sera obtained for diagnostic purposes was approved under the Research Code of the Academic Medical Center, which states that research on biological material is permitted when privacy is guaranteed, unless the patient has objected.

### Cell lines and viruses

The neutralization assays were set up type-specifically using HT-29 and VeroE6 cell lines (American Type Culture Collection, ATCC). Cells were maintained in DMEM supplemented with 10% foetal bovine serum (FBS) and 100 IU/ml of penicillin and 100  $\mu\text{g}/\text{ml}$  of streptomycin in  $37^{\circ}\text{C}$  humidified atmosphere with 5%  $\text{CO}_2$ . Recently isolated Finnish

and Dutch HPeV strains, which induced a clear CPE in cell culture, were used to validate the assays. The Dutch strains 152212 (HPeV1) [16], 751312 (HPeV2) [27], K251176-02 (HPeV4) [5], 20552322 (HPeV5) [16], and 20751393 (HPeV6) [12] were isolated from clinical specimens and the FI0688 (HPeV3) strain was isolated from a healthy child in Finland [22]. In addition, the HPeV1 Harris strain was used for a subset of samples.

### **Determination of neutralizing antibodies by microneutralization assay**

A microneutralization assay was set up for HPeV types 1 to 6 using virus titres of 50 TCID<sub>50</sub> per 2.5 µl. VeroE6 cells were used for the assay of HPeV3 antibodies, whereas HT29 cells were used for HPeV types 1, 2, 4, 5 and 6. Cells were grown to a confluent monolayer on microtiter plates in DMEM containing 10% FBS. Four-fold dilutions of serum (1/16-1/16384) in Hank's balanced salt solution (including CaCl<sub>2</sub> and MgCl<sub>2</sub>) were prepared. A mixture of serum dilution (2.5 µl) and virus (2.5 µl) was incubated for 1 h at 37°C and subsequently overnight at RT before inoculation to the cells. The cells were then grown in medium (DMEM + 2% FBS) for 5-7 days prior to staining with crystal violet. Unlike other types, HPeV3 infection only turned the cells dark and round shaped (CPE) instead of detaching them from the bottom of the well and thus the CPE was observed by a light microscope. Antibody titre was considered to be the highest serum dilution able to prevent 50% or more of the infection. The lowest titre counted as positive was 16. Paired serum samples of 12 patients were tested for each nABs to validate the assay and a subset of 181 samples were analysed for nABs against the HPeV1 Harris strain, to test if results are dependent on the virus isolate used.

### **Determination of antibody response in five children after HPeV infection**

Available sera of five children with confirmed HPeV infections (virus isolated from stool) were tested for nABs. Three of the children had HPeV3 infection and two HPeV6 [22]. Neutralizing antibody positivity was tested from serum samples collected of each child before and after the date of virus detection.

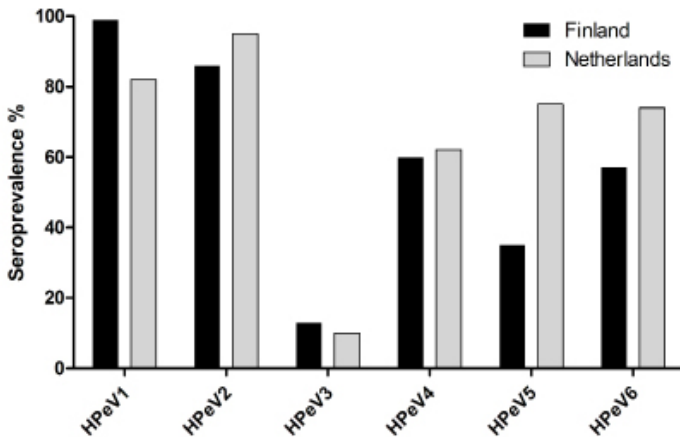
### **Statistics**

IBM SPSS statistics 19 was utilized to perform a Pearson Chi-squared to calculate significance between different groups within our study population. P values less than 0.05 were considered significant.

## Results

### Seroprevalence of HPeVs in Finland and the Netherlands

Seroprevalence of HPeV types 1-6 was measured by neutralizing antibody detection from sera of adult Finnish and Dutch populations. Seropositivity was high for HPeV1 (99% positivity in Finnish samples and 82% in Dutch samples) and HPeV2 (86% and 95% respectively, Fig. 1). In contrast, nABs against HPeV3 were detected only in 13% of the Finnish and 10% of the Dutch samples. Seroprevalence of HPeV4 was similar in both populations (60% in Finland and 62% in the Netherlands) whereas the seroprevalence of HPeV5 (75% vs. 35%;  $p < 0.001$ ) and HPeV6 (74% vs. 57%;  $p = 0.04$ ) was significantly higher in the Dutch adult population compared to the Finnish.

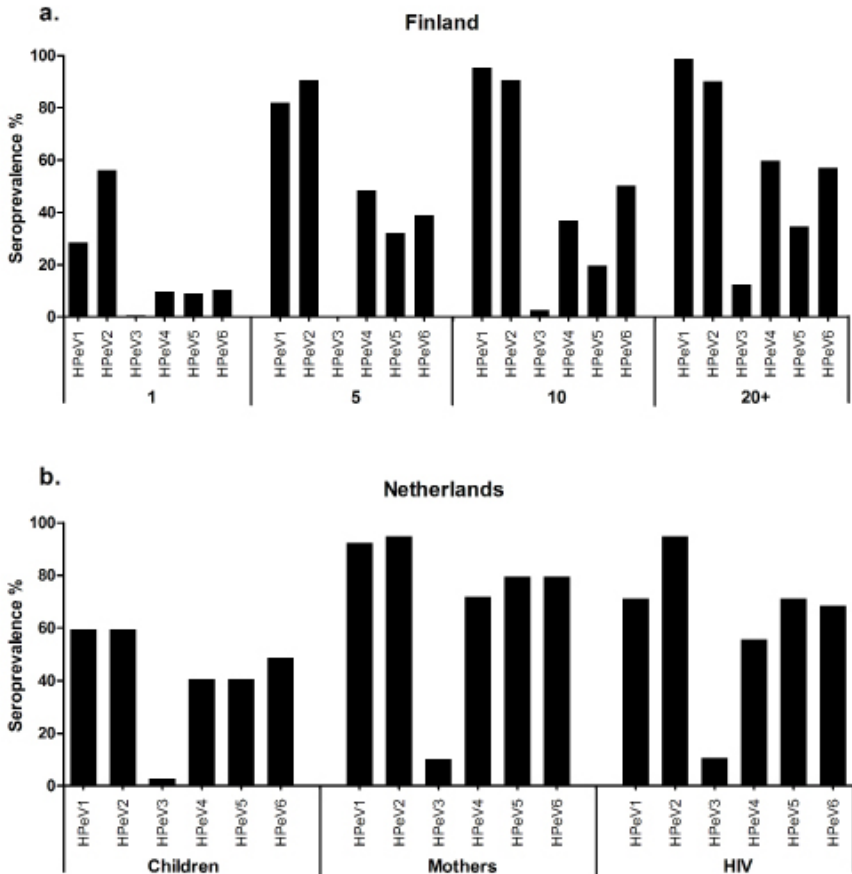


**Fig. 1.** Seroprevalence among adults in Finland and the Netherlands Seropositivity of adults in Finland ( $n = 72$ , black bars) and adults in the Netherlands ( $n = 77$ , grey bars).

### Seroprevalence in subpopulations

Next, the seroprevalence of HPeVs was analyzed in different subpopulations. The Finnish study population was divided into four groups: children aged 1, 5, and 10 years and adults. The Dutch population was divided into three groups: children, women in childbearing age, and HIV-positive men. At the age of one year, Finnish children were antibody positive for HPeV1 in 27% of samples, while already 56% of the samples were positive for HPeV2 and only a small percentage was seropositive for HPeV types 4, 5, and 6 ( $< 12\%$ ). At the

age of 5, the seroprevalence of HPeV1 and HPeV2 increased to 83% and 91% (Fig. 2a). At the age of 10 years, the seroprevalence for HPeV1, HPeV2 and HPeV6 were similar to the seropositivity in adults while the seropositivity for HPeV4 and HPeV5 were still lower compared to the adult population (Fig. 2a).



**Fig. 2.** Seroprevalence of HPeV1-6 in the sub populations. (a) Seropositivity of HPeV in Finland, the Finnish study population was divided into four groups: children aged 1 ( $n = 144$ ), 5 ( $n = 149$ ), and 10 ( $n = 147$ ) years and adults ( $n = 72$ ). (b) Seropositivity of HPeV in the Netherlands, the Dutch population was divided into three groups: children ( $n = 37$ ), mothers ( $n = 39$ ), and HIV-positive men ( $n = 38$ ).

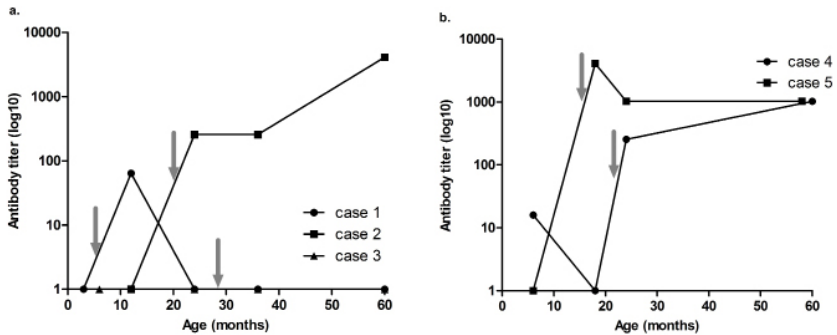
For HPeV3, the seropositivity was low in all three age groups (<2.7%, Fig. 2a). There were no significant differences between boys and girls (data not shown). Among the Dutch children, 60% were positive for HPeV1 and HPeV2, and almost half of Dutch children showed seropositivity for HPeV types 4, 5, and 6 (41%, 41% and 49%, Fig. 2b). Similar positivity was detected for HPeV4 (48%) in 5-year-old Finnish children while a lower prevalence existed for HPeV5 (32%) and HPeV6 (39%, Fig. 2a). In the Dutch population, similar to the Finnish population, seroprevalence against HPeV3 was low (2.7%) (Fig. 2a and b). Within the group of children, an increase of seropositivity is shown with age (data not shown). Dutch women in childbearing age showed a significantly higher seroprevalence of HPeV1 compared to HIV-positive men ( $p=0.016$ , Fig. 2b), while seropositivity of types 4, 5, and 6 was comparable (Fig. 2b).

### **Strain dependency of neutralizing antibodies against HPeV1**

We analyzed 181 samples with two HPeV1 strains, the currently circulating strain 152212 and the older Harris strain. The overall prevalence was similar with the strains 97% (175) being positive for 152212 and 95% (171) being positive for Harris. Only minor differences existed between titers of nAb's detected with both strains (data not shown).

### **Neutralizing antibody response after HPeV infection**

Since only a very low percentage of the samples had nAbs against HPeV3, the neutralizing Ab response after HPeV3 infection was studied. Serum samples from three children with proven HPeV3 infection (HPeV3 isolated from stool) were tested for the presence of HPeV3 nAbs (Fig. 3a). In the first case, an antibody rise was detected, but the antibody titer decreased again and disappeared within 20 months. The second case showed a rise of antibodies, which remained elevated for up to 60 months. Case 3 did not develop any nAbs against HPeV3 after infection. As a control, two HPeV6 positive (HPeV6 isolated from stool) children were tested for HPeV6 nAbs. Both children showed an increase in antibody titres, which remained elevated for >60 months (Fig. 3b). One of the HPeV6 positive children had maternal antibodies in the first follow-up sample.



**Fig. 3.** Antibody response in children after HPeV3 or HPeV6 infection. (a) Longitudinal follow-up serum samples from three cases with a HPeV3 infection confirmed by virus isolation from stool were tested for neutralizing antibody titres at different time points. (b) As a control longitudinal serum samples two sera from two cases with an HPeV6 infection detected in stool were tested for nAb titres at different time points. In one child, maternal antibodies are present in the first follow-up sample. Time of infection is indicated with arrow.

## Discussion

Here, we describe nAb positivity against six HPeV genotypes in Finnish and Dutch populations. High nAb positivity was found for HPeV1 and HPeV2 (82%-99%) in the total adult population in both countries, which is in accordance with previous studies showing, among adults, a high seroprevalence of HPeV1 ranging from 97-99% [24,25]. More than half of the children, aged up to 5 years, were HPeV1 nAb positive, which correlates with previous studies in which HPeV1 infection exists in this age group. The total seroprevalence of HPeV5 and type 6 was higher within the Dutch population compared to the Finnish population. This is in line with the previously observed lower prevalence for HPeV types 4, 5 and 6 in Finland compared to the Netherlands [12,22]. As expected the seroprevalence of all HPeVs increased with age and reached its maximum already in the subpopulation of 10 year-old children. Enterovirus infections have been shown to be more frequent among boys than girls [28]. We, however, observed seroprevalence of HPeV to be equally common in both sexes (data not shown).

Surprisingly, for HPeV2, a high Ab positivity was observed already at the age of 1, while HPeV2 viruses have been detected very rarely in Finland and the Netherlands [12,22]. Large collections of stool samples were screened in many studies, especially from young children, and HPeV2 were detected rarely [12,22,27,29], which makes it unlikely that



HPeV2 infections are missed because infections are mild and patients are not admitted to the hospital. We found consistently high seroprevalence against HPeV2, raising the issue whether cross-reactivity exist among HPeVs. Joki-Korpela *et al.*, (2000) showed weak cross reactivity of HPeV1 and the RGD containing CAV9 [30]. Moreover, they described more dominant antigenic sites at the N-terminal region of VP0 next to the RGD motif [30], suggesting the presence of different (cross-) reactive nAbs against each genotype. Therefore, it cannot be excluded that the high seroprevalence against HPeV2 could in fact be caused by cross reactive nAbs against other RGD containing picornaviruses such as HPeV1 or coxsackievirus A9.

For HPeV3, a study from Japan reported a seroprevalence of 68% among 207 individuals aged from 7 months up to 40 years [8]. In contrast, we observed a very low nAb positivity against HPeV3, 4% in Finland and 8% in the Netherlands. This was unexpected since in both countries HPeV3 is detected frequently [12,22]. The differences in seroprevalence might be explained by different time of introduction of HPeVs in the population. The recent discovery of HPeV3 [8] suggests that HPeV3 is a new circulating HPeV type in both countries. Thus, immunity against HPeV3 could be low. Nonetheless, HPeV types 4, 5 and 6 were discovered later but a high seroprevalence was observed for these three types. A previous time-scale study for HPeV evolution showed that the genetic diversity of the currently circulating types arose around 400 years ago [31]. In addition, the observation of HPeV3, already in 1994 [32] in Dutch stool samples, does not support the hypothesis that HPeV3 is a new viral type. Recently Mizuta *et al.*, reported neutralization in only 5/20 adults with confirmed HPeV3 infection, which supports the general difficulties in the detection of HPeV3 antibodies [33]. Similarly Westerhuis *et al.*, reported lack of nAbs after HPeV3 infection in two donors [34]. Here, we observed that after HPeV3 infection, the nAb rise remained elevated for a longer period in only one out of three cases, and in the third case, no nAbs were detected, while a permanent rise in nAb after HPeV6 infection was shown, in agreement with previous results showing that the nAb levels against HPeV1 retain a permanent increase [25]. This indicates difficulties in nAb production against HPeV3. A possibility is that the low frequency of HPeV3 nAbs detection is a test artifact. HPeV3 is grown on VeroE6, while the other HPeVs grow best on HT29 [34]. It may be that this difference in cell lines influences the neutralization outcome. At the moment, however, methodologies to explore this notion are limited.

In conclusion, our seroprevalence data confirm that HPeVs are among the most commonly occurring viruses both in Finland and in the Netherlands. Similar trends in seroprevalence could be shown in populations from Finland and the Netherlands. However, the selection of the population studies was performed differently and therefore selected

subpopulations from the different countries cannot be compared. To assess seroprevalence in subgroups from different regions, further in-depth studies are needed.

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# 6.

## **Human memory B cells producing potent cross-neutralizing antibodies against Human Parechovirus; implications for prevalence, treatment and diagnosis**

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## Abstract

The family *Picornaviridae* is a large and diverse group of positive sense RNA viruses, including the human enteroviruses (HEVs) and human parechoviruses (HPeVs). The human immune response against HEVs and HPeVs is thought to be mainly humoral and an insufficient neutralizing antibody (Ab) response during infection is a risk factor and can ultimately be life threatening. The accessibility of different antigenic sites and observed cross reactivity makes HPeVs a good target for development of therapeutic human monoclonal antibodies (mAbs). In this study we generated a polyclonal rabbit IgG mixture by rabbit immunization with HPeV1 and two different human monoclonal Abs specific for HPeV. These mAbs were generated by screening culture supernatants of Ab producing human B cell cultures for direct neutralization of HPeV1. Both polyclonal and monoclonal Abs showed specific neutralization, as well as neutralization of HPeV2. HPeV1 mAb AM18 showed cross-neutralization of HPeV4, 5 and 6, and even of the HEV coxsackievirus A9 (CVA9). The Ab cross-reactivity seems to be restricted to strains containing the RGD motif at the C-terminus of the capsid protein VP1. A VP1 specific ELISA confirmed that the AM18 bound the capsid protein of HPeV1, 2 and 4. In contrast, the HPeV1 specific mAb AM28, which neutralized HPeV1 even more efficiently, showed no cross-reactivity with HPeV3-6 or HEVs and did not bind any of the capsid proteins, suggesting specificity for a conformation dependent, non-linear epitope for this Ab. This observed cross reactivity of two monoclonal Abs, gives a good perspective for the development of cross-protective anti- HPeV treatment.

## Introduction

The family *Picornaviridae* is a large and diverse group of positive sense RNA viruses, which consists of several important human and animal pathogens. The family contains 26 genera including the *Enterovirus* and *Parechovirus* genera. The genus *Enterovirus* contains over 250 recognized types that can infect humans, including poliovirus (PV), echovirus (E), coxsackie-A and B viruses (CVA and CBV) and rhinovirus (RV), while the species *Parechovirus* presently contains 16 recognized genotypes (1–8). HEV and HPeV infections are common in young children and adults causing a broad range of disease, including gastrointestinal and respiratory tract infections, aseptic meningitis, paralysis, myocarditis and neonatal sepsis. HEV and HPeV infections can be severe and life threatening, while no antiviral treatment is available. Presence of maternal antibodies (Abs) and Abs transferred during breast feeding play an important role in protection of neonates against picornavirus infection. For HEV it has been shown that in neonates with severe HEV infection, the absence of maternal virus-specific neutralizing Ab titers is correlated with disease, suggesting HEV specific neutralizing Abs are important for protection (9). In neonates with severe HEV infection intravenous immunoglobulins (IVIg) is given as supportive treatment, but high serotype specific Ab titers are needed, which can vary between IVIg batches (10). Therefore, treatment against HPeV viral infections with specific monoclonal antibody (mAb) would be a good option. mAbs are highly specific and normally well accepted by the host. These Abs currently do not exist for HPeV and HEVs but with new approaches to rapidly generate human mAbs, as has been shown for influenza viruses (11) and respiratory syncytial virus (12, 13), this should be feasible. Since infections with HEVs are transient and humoral immunity is responsible for clearing the virus, new vaccination strategies should focus on eliciting neutralizing Abs. For poliovirus very effective vaccines were developed in the 1950s inducing humoral immunity, which prevent viraemia and disease. However the neutralizing Abs against one serotype did not protect against infection with the other serotypes, therefore vaccines had to contain single strains of each serotype (14, 15). Since the capsid of HEVs consist of 60 protomers constructed out of 4 polypeptides namely VP2, VP4, VP3 and VP1 the neutralizing Ab response is directed against these polypeptides. HEVs major neutralizing antigenic sites reside on the VP1 capsid protein and lesser extent on VP2 and VP3 (16–18). In the case of foot and mouth disease (FMDV) virus, another vaccine preventable picornavirus affecting cloven-hoofed animals, a dominant antigenic site is located in the GH loop of the VP1 capsid (19–21). Neutralization directed against this epitope is largely serotype specific and cross-reactivity is limited between serotypes, due to broad antigenic variation (22–26). Thus, neutralizing Abs against picornaviruses are considered genotype specific and it is assumed that they do not cross-protect against infections with other types.

HPeVs show many resemblances to HEVs with respect to clinical manifestations as well as



biological features (27). An important difference is that VP0 is not cleaved into VP4 and VP2 in HPeVs and little is known about humoral immunity against HPeVs. Two studies from Finland show seroprevalence >97% for HPeV1 in adults (28, 29). In a combined study in Finland and the Netherlands a seroprevalence of neutralizing Abs in adults was high for HPeV1 (>92%), and for HPeV2 (>86%) (30). Interestingly the response against HPeV2 cannot be explained by circulating HPeV2 virus since it is not detected in these countries. The responses are directed against different antigenic sites in the three different capsid proteins of HPeV namely VP0, VP3, and VP1 (31). In a study of Alho *et al.*, (32) potent neutralization could be obtained with serum from rabbits vaccinated with HPeV-VP1 protein against VP1, and it was shown that the C-terminal region of VP1 is an important antigenic site. In addition, antiserum elicited against VP0 could partly neutralize HPeV1 infection, showing that the N-terminal region of the VP0 is another antigenic site. While the humoral immune response against HEVs is thought to be type specific, some cross-neutralization has been shown. CVA9 antiserum showed full inhibition of HPeV1 infection in cell culture, and HPeV1 antiserum almost completely blocked CVA9 infectivity. Both viruses contain the arginine-glycine-aspartic acid (RGD) containing C-terminal VP1 polypeptide, and after deletion of the RGD motif in CVA9 this cross neutralization was diminished (32). HPeV1, HPeV2, 4, 5, and 6 contain a similar RGD motif, and indeed, cross-neutralization has been reported with polyclonal Abs against these viruses (33).

Since the absence of neutralizing Abs is a risk factor for severe disease in infants, prophylactic and possible therapeutic treatment of picornavirus infections with mAbs would be a logical option. The accessibility of different antigenic sites, and the observed cross reactivity makes the small group of HPeVs a good target for development of therapeutic human mAbs. In this paper, we describe the characteristics of one polyclonal, and two novel HPeV1 cross-neutralizing human mAbs.

## Material and Methods

### Virus culture and purification

HPeV1-Harris and the HPeV2-2008 strains were provided by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands, the primary strains HPeV3-150237, HPeV4-251176, HPeV5-552322 and HPeV6-550389 were obtained in house (34, 35). For testing cross neutralization the following HEV strains were used: EV71, CVB3, RVA16, CVA9-AMC, CVA9-1051883, CVA-1052186, E9-1252784, and E9-Barty. The RGD-containing E9-Barty strain was a kind gift from Prof. F. van Kuppeveld (Department of Virology at the Faculty of Veterinary Medicine, University of Utrecht). For virus culture of HPeV1, 2, 4, 5, and 6, CVA9 and the E9 strains the human colon carcinoma

(HT29) was used, HPeV3 was cultured on African green monkey kidney (Vero) cells. The cells were maintained in Eagle's Minimum Essential Medium (EMEM) with L-glutamic acid (0.2X), non-essential amino acid (1X), streptomycin (0.1 µg/ml) and ampicillin (0.1 µg/ml), supplemented with 8% heat-inactivated Fetal Calf Serum (FCS). The virus concentration was determined by the median tissue culture infective dose (TCID<sub>50</sub>) and calculated by the Reed and Muench method (30). For total virus purification cells were infected with an MOI of 0.1 and after appearance of CPE (cytopathic effect) grade 4+ (75-100% infection) the supernatant and cells were collected and freeze thawed twice (-80°C) then centrifuged at 4000rpm for 15 minutes. After the supernatant was collected and filtered through a 0.22 µm filter, it was centrifuged at 32000rpm, for 2h at 4°C in the ultracentrifuge (rotor SW32Ti, Beckman). The pellet was dissolved in 1xTNM buffer (10mM Tris-HCl, pH 7.5, 150mM NaCl, 1mM MgCl<sub>2</sub>) and virus was purified on a cesium chloride step gradient made of 40% (w/v) bottom layer, 15% (w/v) top layer by centrifuging at 32000rpm, for 16hrs at 4°C (rotor SW41Ti, Beckman). The fraction containing the virus was harvested and exchanged with TNM buffer and concentrated with 100kDa cutoff filter (Millipore).

### **Rabbit polyclonal Ab preparation and human monoclonal Ab discovery**

Polyclonal Abs were obtained by Rabbit immunization (Harlan Laboratories). The purified virus was used for immunization of one rabbit. The rabbit received 5 times every 14 days a boost of 200µg purified virus and nancy. The final bleed was taken after 77 days.

The AM18 and AM28 Abs were obtained from healthy human donors using a direct virus neutralization assay of HPeV1 virus pre-incubated with Ab containing supernatant of cultures with different B cell densities, generated as described previously (12, 13). In brief, CD27+ memory B cells were isolated from peripheral blood by FACS sorting. Cells were stimulated for 36 hrs with CD40L and interleukin (IL)21 and subsequently transduced with a retrovirus containing the Bcl-6 and Bcl-xL transgenes together with the marker gene GFP. Transduced B cells can be maintained for prolonged periods of time and harbor a Germinal Center phenotype, which is characterized by expression of cell surface immunoglobulin (the B Cell Receptor -BCR-) and secretion of soluble immunoglobulin. Abs present in the supernatant can be tested for binding to specific antigens or tested in direct functional assays. Here we tested the HPeV1 neutralizing capacity of Abs in B cell supernatants by co-incubation of supernatants with virus (at 100TCID<sub>50</sub>/ 50µl and 20TCID<sub>50</sub>/ 50µl) for 1 hr before they were added to HT29 cells. Infection was determined by read out of CPE. Cultures that blocked CPE formation were single cell subcloned to obtain monoclonal B cell cultures. The Ab heavy and light chain genes were retrieved from these B cell clones and expressed as recombinant protein in 293T cells. IgG1 Abs were subsequently purified using HiTrap Protein A columns on an ÄKTA instrument (GE).

### Virus neutralization assay

To determine neutralizing potency the AM18 and AM28 Abs were tested in an endpoint neutralization assay starting at 25µg/ml. Abs were mixed with HPeV1 virus suspension containing 100 TCID<sub>50</sub>/50µl. Mixtures were incubated at 37°C for 1hr before inoculation of HT29 cells in a 96-wells plate (200 µl). As positive control, cells were inoculated with 100TCID<sub>50</sub> with an irrelevant Ab and without Abs. As negative controls only Abs or only medium was added to the cells. The cells were examined for the appearance of CPE every 24h for 7 days. At day 7, 25µl of supernatant was removed for RNA isolation using the total nucleic acid isolation kit with the MagnaPure LC instrument® (Roche Diagnostics). The RNA was eluted in 50µl elution buffer and reverse transcribed as previously described (34). Five µl of cDNA was used for to estimate viral copy number using LC480® real time PCR machine (Roche Diagnostics). The virus copies per PCR were calculated with a standard curve as previously described (36).

### Expression and purification of HPeV capsid proteins

To generate recombinant capsid protein VP0, VP3 and VP1 from the HPeV1-Harris cDNA strain and the virus isolates HPeV2, 3, 4 primer sequences were used as depicted in Table S1, using the following PCR: 5' 95°C, followed by 35 cycles 30s at 95°C, 30s at 55°C and 60s at 72°C and an extension step of 10' at 72°C. The purified PCR fragments were cloned into the expression vector Pet102 with his-tag and expressed in *Escherichia coli* BL21 Star™(DE3) One Shot® cells. Single bacterial colony was inoculated in LB medium supplemented with 100 µg/ml ampicillin and propagated at 37°C with 220rpm speed on a shaker incubator until the culture reached logarithmic growth phase (at OD<sub>600</sub> 0.6–0.7). To stimulate recombinant protein expression 0.5mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to the cultures. The proteins were purified by using Ni-NTA purification system with Anti-His(C-term)-HRP Ab in accordance with the manufacturer's instructions (K953-01, Invitrogen).

### ELISA

ELISA plates were coated with 200ng of purified HPeV1-6, or the different capsid proteins VP0, VP3, VP1 from HPeV1-4 overnight at room temperature (RT). After washing with 0.1%tween/PBS (3 times) the protein coated ELISA plates were blocked with 2% BSA in PBS for 2 hrs at room temperature. The plate was incubated with 2 µg/ml of aHPeV1 AM18 or AM28 for 1 hr at RT and washed 3 times with PBS/0.1% Tween. Anti-human or anti-rabbit IgG, HRP labeled (0.3 µg/ml) was used as the secondary Ab, incubated for 1 hr at RT and washed three times with PBS/0.1% Tween. The substrate solution containing 3,3',5,5'-tetramethylbenzidine was added and incubated for 10min at RT in the dark. The

reaction was stopped by the addition of 0.8 M H<sub>2</sub>SO<sub>4</sub>. The O.D. at 450nm and 620nm was measured with a microplate reader.

### Western blot

The whole virus and purified capsid proteins VP0, VP3 and VP1 were run on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in duplicate. One gel was stained with coomassie brilliant blue R/G and the other was used for western blotting using semi dry blotting at 15V for 15 min on a nitrocellulose membrane. The membrane was blocked overnight at room temperature (RT) with 5% donkey serum in PBS. HPeV1 specific mAbs AM18 and AM28 were diluted in washing buffer, containing 1% donkey serum in 0.1% Tween20/PBS and added to the membrane. The membrane was washed 3 times for 5 min each, and incubated with anti-human IgG Alexa 486 (1:5000 dilution) in washing buffer for 1hr at RT. The membrane was washed 3 times and scanned with the Odyssey infrared imaging system (LI-COR, biosciences).

### Immunofluorescence assay

The immunofluorescence was performed as described previously (33). In brief, HT29 cells were incubated with the primary Abs AM18 (2 µg/ml) and AM28 (2 µg/ml). As secondary Ab 15 µg/ml of goat anti-human IgG- Alexa 488 was used and the nuclei was stained with 0.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma).

## Results

### Generation of HPeV1 neutralizing mAbs

Human memory CD27+IgG+ B cells were obtained from peripheral blood from three healthy donors and were transduced with Bcl-6 and Bcl-xL and cultured at 50 cells per well in 96 well plates before they were frozen at -150°C in a 96 well plate format. The corresponding B cell culture supernatants were also frozen thereby creating a B cell and culture supernatant biobank. The supernatants were tested for direct neutralization of 100 and 20TCID<sub>50</sub> of HPeV1 in a HT29 cell rounding assay at the appropriate time. From the cultures that showed reduced cell rounding at both TCID<sub>50</sub>s, single B cell cultures were generated to retrieve the original monoclonal B cell. From clones that showed repetitive neutralization of HPeV1, RNA was isolated to retrieve the Ab heavy and light chain sequences (Table 1, Supplementary Table 1). These sequences were used to generate recombinant protein from 293T cells. Ab AM18 (original clone 18E10, VH1-3) and AM28 (original clone 28G3, VH3-23) were both of the IgG1 isotype and both contained a Kappa light chain. The B cells

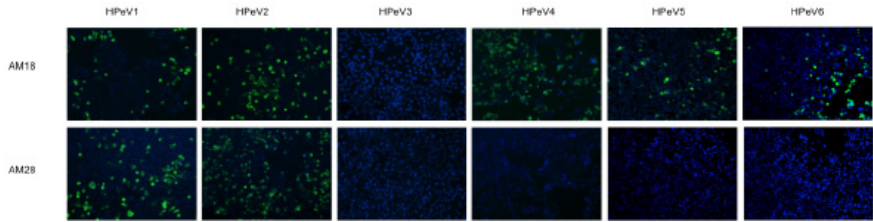
presumable have gone through multiple rounds of selection and somatic hypermutation since the VH contained up to 22 amino acid substitutions for AM18 and 16 for AM28. In addition to the VH chain, selection has also been imposed on the light chain with 6 amino acid substitutions in each. Based on the completely different VH and VL gene usage the B cell clones do not seem to be related.

**Table 1.** mAb characteristics

Clone	Isotype	IGHV	IGHD(frame)	IGHJ	CDR3H	aa replacement
						VH
AM18	IgG1	1-3*01	4-23*01	4*02	GNFGGNSATFDY	22
AM28	IgG1	3-23*01	3-10*01	4*02	DGSGSHYNNNYFDS	16
		IGKV/LV	IGK/LJ		CDR3L	VL
AM18	κ	3-20*01	4*01		QQYGGSP	6
AM28	κ	3-15*01	2*01		QQYNNWP	6

### Cross reactivity of HPeV1 specific polyclonal and human mAbs between HPeVs

The polyclonal rabbit Ab aHPeV1-HARLAN and the monoclonal Abs AM18 and AM28 both neutralizing HPeV1, were tested for binding to HPeV1 to 6 viral proteins in infected cells, by immunofluorescence. All Abs showed specific binding to HPeV1 and HPeV2. In addition, the aHPeV1-HARLAN and AM18 Ab were specifically recognizing cells infected with HPeV4, 5 or 6 (Figure 1). None of the mAbs did bound to HPeV3- or non-infected cells. To identify capsid protein specificity, an ELISA was performed with whole purified virus and the different recombinant capsid proteins of HPeV1. The aHPeV1-HARLAN polyclonal Ab mixture showed binding to all the genotypes and recognized the capsid proteins VP0 and VP1 of HPeV1-4. AM18 showed binding to whole purified virus of HPeV1, 2, 4, 5, and 6, and showed binding to the (denatured) VP1 protein of HPeV1, 2, and 4 (Table 2). As shown in the immunofluorescence assay the AM28 Ab recognized HPeV1 and HPeV2 whole purified virus, but did not recognize any of the capsid proteins by ELISA (Table 2). All these data were confirmed by western blot (data not shown).



**Figure 1.** Detection of AM18 and AM28 binding to HPeV1-6 infected cells by Immuno-fluorescence staining. HT29 cells were used for infection with HPeV1, 2, 4, 5 and Vero cells for HPeV3. When CPE was observed the cells were fixed with 4% paraformaldehyde and stained with the monoclonal Abs, and binding was detected with a secondary goat anti human IgG-Alexa-488 labeled (Green). Nuclei were detected using DAPI.

**Table 2.** Binding of HPeV1 polyclonal Ab and monoclonal Abs to purified HPeV1-6 and the different capsid proteins in an ELISA

		Strain	aHPeV1-HARLAN	aHPeV1-AM18 Ab	aHPeV1-AM28 Ab
Purified virus		HPeV1-Harris	+	+	+
		HPeV1B	+	+	+
		HPeV2	+	+	+
		HPeV3	+	-	-
		HPeV4	+	+	-
		HPeV5	+	+	-
		HPeV6	+	+	-
Capsid proteins	HPeV1	VP0	+	-	-
		VP3	-	-	-
		VP1	+	+	-
	HPeV2	VP0	+	-	-
		VP3	-	-	-
		VP1	+	+	-
	HPeV3	VP0	+	-	-
		VP3	-	-	-
		VP1	+	-	-
	HPeV4	VP0	+	-	-
		VP3	-	-	-
		VP1	+	+	-

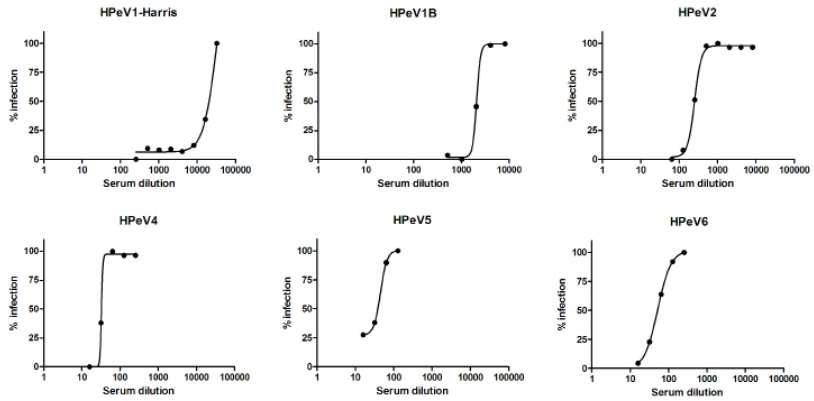
### Ab neutralization of HPeV1 to 6

The HPeV1 Abs were tested for neutralization capacity *in vitro* against 100TCID<sub>50</sub> of HPeV1 to 6 by monitoring inhibition of CPE as well as decrease in copy number by real time PCR (Figure 2, 3, 4). The HPeV1 Ab AM18 strongly neutralized the HPeV1-Harris and HPeV1B strains at IC<sub>50</sub> values of 5 ng/ml (Table 3, Figure 3). The HPeV1 Ab AM28 neutralized the HPeV1-Harris and HPeV1B strain even better at IC<sub>50</sub> values of 3 ng/ml (Table 3, Figure 4). In addition, both Abs showed strong cross-neutralization against HPeV2 ranging from 32-78 ng/ml (Table 3, Figure 3, 4). In addition the polyclonal and the monoclonal AM18 Ab but not AM28 could neutralize HPeV4, 5 and 6 at concentrations ranging from 295-555 ng/ml respectively (Table 3, Figure 3, 4, 5). No cross- reaction in binding or neutralization with HPeV3 was observed.

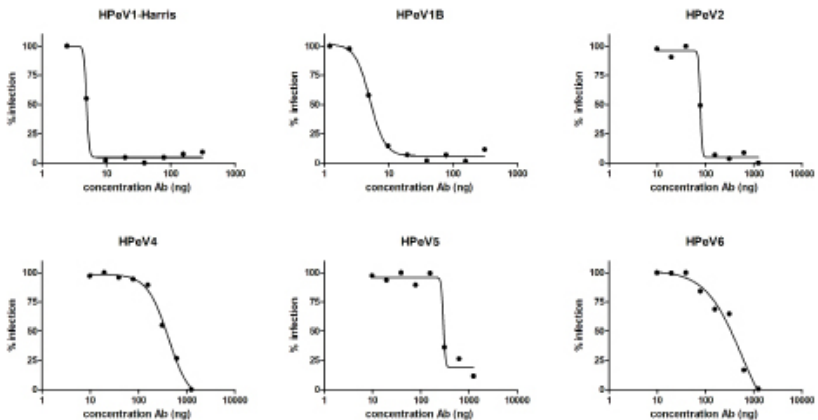
**Table 3.** Neutralization IC<sub>50</sub> values for HPeV1 monoclonal Abs

	Neutralization IC <sub>50</sub> aHPeV1-AM18		Neutralization IC <sub>50</sub> aHPeV1- AM28	
	titer	ng/ml	titer	ng/ml
<b>HPeV1-Harris</b>	4076	4.9	3650	5.5
<b>HPeV1B</b>	3888	5.1	7580	2.6
<b>HPeV2</b>	256.6	78	615.7	32.48
<b>HPeV3</b>	ND	ND	ND	ND
<b>HPeV4</b>	47.82	418	ND	ND
<b>HPeV5</b>	67.8	295	ND	ND
<b>HPeV6</b>	36.1	555.3	ND	ND
<b>CVA9-amc</b>	117.6	170	ND	ND
<b>CVA9-1051883</b>	63.39	315.5	ND	ND
<b>CVA9-1052186</b>	ND	ND	ND	ND
<b>Echo 9-amc</b>	ND	ND	ND	ND
<b>Echo 9-Barty</b>	ND	ND	ND	ND
<b>Echo 9-1252784</b>	ND	ND	ND	ND

ND = not detected

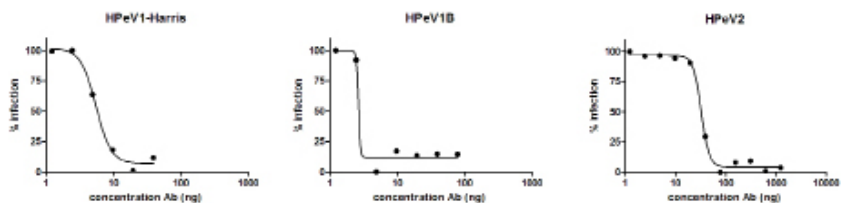


**Figure 2.** Neutralization of HPeV1 to 6 using the polyclonal rabbit antiHPeV1-HARLAN serum. An end point neutralization assay is performed with 100TCID<sub>50</sub> of HPeV1-6. The viral copies per PCR are measured at day 7 at the different serum dilutions. The IC<sub>50</sub> curves are generated with Graphpad Prism 5. Experiments are performed two or three times, the datapoints are the average of the different experiments.



**Figure 3.** Neutralization of HPeV1 to 6 using the monoclonal AM18 Ab. An end point neutralization assay was performed with 100TCID<sub>50</sub> of HPeV1-6. Presence of HPeV RNA was detected at day 7 after infection in HT29 cell supernatants by PCR. The IC<sub>50</sub> curves are generated with Graphpad Prism 5. Experiments are performed at least twice two or three times, the data points are the average of the different experiments.

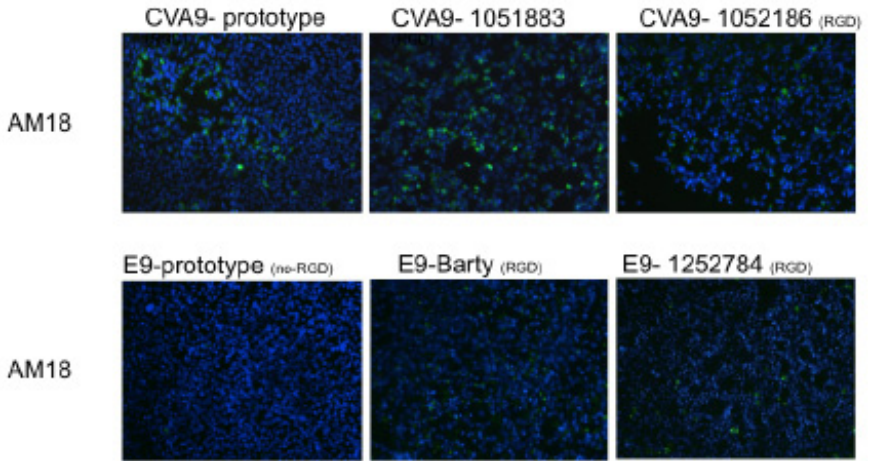




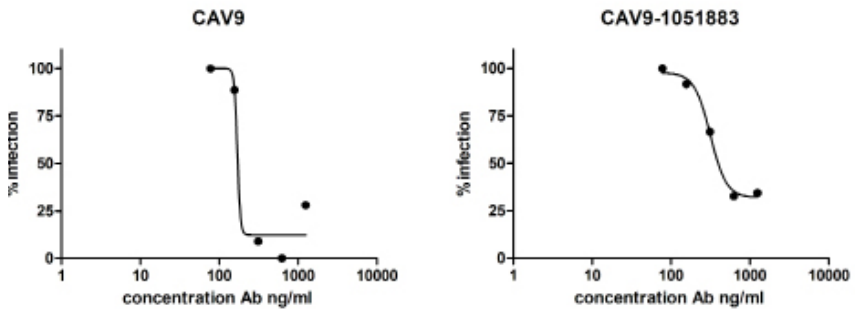
**Figure 4.** Neutralization of HPeV1 to 6 using the monoclonal AM28 Ab. An end point neutralization assay is performed with 100TCID<sub>50</sub> of HPeV1-6. The viral copies per PCR are measured at day 7 at the different Ab dilutions (titers). The IC<sub>50</sub> curves are generated with Graphpad Prism 5. Experiments are performed at least twice, the datapoints are the average of the different experiments.

### Ab cross reactivity with HEVs

To test whether the aHPeV mAbs were genus-specific only, both mAb AM18 and AM28 were tested for neutralization of other HEVs. The AM18 and AM28 did not neutralize the non RGD-containing HEVs EV71, CVB3, or RVA16. However, since cross reactivity of polyclonal HPeV1 Abs with the RGD-containing CVA9 has been reported (15), AM18 and AM28 specificity to different CVA9 strains (CVA9- AMC, and clinical strains 1051883, 1052186) as well as an RGD-containing (E9-Barty) and a non-RGD containing E9 strain (clinical strain 1252784) was tested with an immunofluorescent assay and standard neutralization assays. AM18 was specifically recognizing all three RGD-containing CVA9 strains, although weakly CVA9-1052186 (Figure 5). Similar results were obtained in the neutralization assays, where the CVA9 prototype was neutralized at an IC<sub>50</sub> of 118 ng/ml and an IC<sub>50</sub> of 63 ng/ml was found for the clinical isolate 1051883, while we could not detect Ab induced inhibition of isolate 1052186 (Figure 6). In case of the E9, the non-RGD nor the RGD containing E9 strains were neutralized by AM18 or AM28 (data not shown), although some weak binding could be found for the AM18 with the E9-1252784. The RGD surrounding amino acids could influence Ab binding to the RGD motif, since the sequences show relatively high similarity in the RGD region (Figure 7).



**Figure 5.** Immuno-fluorescence showing AM18 binding to infected HT29 cells with our prototype CVA9, and clinical strains 1051883, 1052186 as well as an RGD-containing (E9-Barty and clinical strains 1252784) and a non-RGD containing E9. When CPE is observed the cells are stained with the monoclonal Ab, and secondary Alexa-488 labeled IgG (Green). Nuclei were detected using DAPI.



**Figure 6.** IC50 curves of the monoclonal AM18 Ab. An end point neutralization assay is performed with 100TCID50 of CVA9. The viral copies per PCR are measured at day 7 at the different Ab dilutions (titers). The IC50 curves are generated with Graphpad Prism 5.

```

*HPEV1_HARRIS      : PNFFFPLPAPKVTS---SRAIRGDMANLNTNQ--- :
*HPEV1_450343     : PNFFFPLPAPKVTT---GRAIRGDMANFNSNE--- :
*HPEV1_550163     : PNFFFPLPAPKVTT---SRAIRGDIANFSDQ--- :
  HPEV1_K6394      : PNFFFPLPAPKVTT---GRAIRGDIANFSDQ--- :
*HPEV1_252581     : PNFFFPLPAPKVST---SRAIRGDIANFSDQ--- :
  HPEV2_WILLIAMSON: PNFFFPLPAPKPAT---RKY-  RGDIATWSDQ--- :
*HPeV2_2008       : PNFFFPLPAPKPAT---SKY-  RGDIATWSDQ--- :
*HPEV3_152037     : PNFFFVVPAPKPTG---SRA-----AALYDE--- :
  HPEV3_450936     : PNFFFVVPAPKPTG---SGA-----VALYDE--- :
*HPEV3_A308-99    : PNFFFVVPAPKPTG---SRA-----TALSDE--- :
  HPEV3_651689     : PNFFFVVPAPKPTG---TRA-----IAFHDE--- :
*HPEV4_K251176    : PNLFFPLPAPKPAT---SRAIRGDMANFSDQ--- :
  HPEV4_T75-4077   : PNLFFPLPAPKPTS---GRSIRGDMANFSDQ--- :
  HPEV5_CT86-6760  : PNLFFPSPAPKEKT---SRAIRGDIANFIDQ--- :
  HPEV5_T92-15     : PNLFFPSPAPKEKT---SRTIRGDMANLNTNQ--- :
*HPeV5_552322     : PNLFFPLPAPKPKT---GRAIRGDMANFSDQ--- :
  HPeV6_NII561-200: PNFFFVVPAPKNTPRSQSRRAIRGDMANLNTNQ--- :
*HPeV6_550389     : PNFFFVVPAPKNTPRSQNRRAIRGDMANLNTNQ--- :
  HPeV6_BNI67-03   : PNFFFVVPAPKNTPRSESRRAIRGDMANLNTNQ--- :

*CAV9-amic        : PITDTRKDINTVTTVAQRR-IRGDMSTLNTHGAF :
*CAV9-1052186     : PITDTRKDINTVTSVAQGA-IRGDI AALNTHGVF :
*CAV9-1051833     : PITDTRKDINTVTTIVQSG-IRGDI AALNTHGAF :
*Echo9-amic       : AVTDTRDTINTVPLSTHGVSE---GAYGHQSGAA :
*Echo9-1252784   : AVTDTRDTINTVPVSSHGGARGDIAATLSTRGAF :
*Echo9-Barty      : AVTETRDTINTVPVSNHGSGRGDIAALSTHGAF :

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**Figure 7.** Amino acid alignment of RGD surrounding amino acids at the C-terminus of the VP1 region of the different HPEV1, CVA9 and Echo9 strains.

## Discussion

In this study we generated two unique HPeV1 monoclonal Abs using stable Ab producing B cells by genetic programming (12, 13). We showed that both Abs discovered by directly screening for HPeV1 neutralization, also neutralized HPeV2, indicating that HPeV1 and 2 share similar neutralizing epitopes. This high cross-reactivity of both Abs with HPeV2 could explain why high seroprevalence against HPeV2 is found in the Netherlands and Finland (86-99%) (30), while HPeV2 infections are hardly detected (35, 37, 38), suggesting cross protection and seroprevalence by HPeV1 Abs. In addition, the HPeV1 specific AM18 Ab also cross-neutralized HPeV4, 5 and 6, but not HPeV3. The epitope of AM18 is located in

the VP1 protein since we could detect binding to the recombinant VP1 capsid protein of HPeV1, 2 and 4, but not to the VP0 and VP3 proteins. AM18 is possibly raised against a linear epitope containing the RGD motif, which is well conserved in the genotypes HPeV1, 2, 4, 5, and 6 at the C-terminus of the VP1 capsid protein but also in CVA9, but not in HPeV3. Cross-binding to the RGD motif was shown previously for CVA9 and HPeV1 with polyclonal Abs (31), but it was never thought that this feature could be assigned to a single mAb. The cross-neutralization of the non-HPeV1 genotypes and the EV CVA9 was less efficient, and the RGD-containing E9 strains could not be neutralized, which indicates importance of the RGD surrounding amino acids making the RGD containing motif less accessible for AM18 and/or the total conformation of the VP1 capsid protein differs. In contrast to AM18, we could not specifically determine the target recognized by AM28, which suggests that AM28 is binding to a non-linear, conformation dependent epitope. The AM28 Ab neutralization might block receptor binding, but possibly the Ab may neutralize by inducing conformational changes or interfering with viral uncoating (39–41). For characterization of the exact neutralizing epitopes structural studies of the Ab-virus complex are currently ongoing. The development of a protective B cell response against HEVs is presumably achieved by generation of type-specific Abs against the VP1 capsid protein. However since we discovered two cross-reactive Abs next to the general VP1 specific response a more broad and diverse Ab repertoire may exist that could influence serotyping and prevalence studies based on serum binding assays. Furthermore these cross binding and neutralizing Abs may allow for the development of anti- HPeV treatment and may facilitate vaccine development. It should be noted that our monoclonal Abs did not give any cross-reactivity with the HPeV3 genotype, which already has been shown to differ in many aspects from HPeV1, 2, 4, 5 and 6 (reviewed in (27)). Clinically HPeV3 differs from the other HPeV strains since it is infecting younger children and is more often associated with severe disease, suggesting different receptor use as well as different neutralization mechanisms. Thereby the detection of neutralizing Abs against HPeV3 in serum was shown to be difficult (30, 33), leaving a challenge to find protective HPeV3 mAb in the future.

### Acknowledgements

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# 7.

## **Structural basis of human parechovirus neutralization by human monoclonal antibodies**

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*Submitted for publication*



## Abstract

Human parechoviruses are one of the leading causative agents of neonatal sepsis. Intravenous immunoglobulin treatment is the only treatment available in such life-threatening cases and has given moderate success. Direct inhibition of parechovirus infection using monoclonal antibodies is an alternative treatment. We have developed two therapeutic monoclonal antibodies against human parechovirus 1, namely AM18 and AM28. Here we present the mapping of their epitopes and the mode of their neutralization using peptide scanning, electron cryo-microscopy and fluorescence-based thermal shift assays. We determined by peptide scanning that AM18 recognizes a linear epitope motif including the 'arginine-glycine-aspartic acid' on the C-terminus of capsid protein VP1. This epitope is normally used by the virus to attach to host cell-surface integrins during entry. Therefore, AM18 is likely to cause virus neutralization by blocking integrin binding to the capsid. In contrast, we show by electron cryo-microscopy, three-dimensional reconstruction and pseudo-atomic model fitting that AM28 recognizes quaternary epitopes on the capsid composed of VP0 and VP3 loops from neighbouring pentamers. The melting temperature of the virus-AM28 complex increased significantly compared to the virus alone in thermal shift assays indicating stabilization of the capsid by the antibody, thereby preventing virus uncoating. Thus both mAbs show therapeutic promise for HPeV infections.

## Introduction

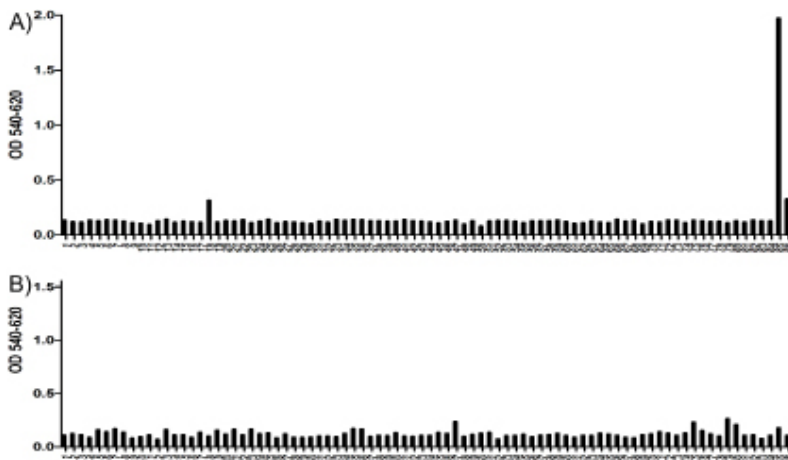
Human parechoviruses (HPeV) are single-stranded, positive-sense RNA viruses in the *Parechovirus* genus within the *Picornaviridae* family [1]. HPeV infections mainly cause mild gastrointestinal symptoms, although HPeVs are also associated with more severe central nervous system symptoms, like meningitis and neonatal sepsis [2–5]. The HPeV genome is about 7300 bases in length, enclosed in an icosahedrally-symmetric capsid of 60 copies of each of the three capsid proteins VP0, VP3 and VP1 [1,6]. HPeVs lack the maturation cleavage of the capsid protein VP0 into VP4 and VP2, which is present in most picornaviruses [7]. They have a 30-amino-acid-long extension to the N-terminus of VP3, and a unique non-structural protein 2A, lacking proteolytic activity [8]. HPeV1 contains an arginine-glycine-aspartic acid (RGD) motif close to the C-terminus of VP1 [1]. The RGD motif is found in a number of viral capsid proteins which recognize integrin receptors to gain entry into host cells e.g. coxsackievirus A9 (CVA9), echovirus 9 (Echo9) and foot and mouth disease virus [9–11]. The role of the RGD motif for HPeV1 has been shown through blocking experiments with RGD-containing peptides and mutations of the sequence, where deletion of the RGD motif is lethal [12–15]. Studies of the HPeV1 virion in complex with both  $\alpha V\beta 3$  and  $\alpha V\beta 6$  integrins confirmed that they have overlapping binding sites on the predicted site of the RGD motif on the capsid surface [6]. The RGD motif has also been shown to be an important antigenic site. Diluted antiserum raised against a peptide containing the RGD motif neutralized 51% HPeV1 infections in a plaque assay compared to a background of 1% in the preimmune serum [16]. When the virion has been used as in antigen in rabbits, the immune sera recognize linear epitopes from VP0 and VP3. 100% neutralization has been shown with rabbit sera raised against virions and VP1 [16,17]. There are several potential neutralizing mechanisms for antibodies that bind specifically to viral capsid surfaces e.g. antibodies may neutralize by obstructing a receptor-binding site, cause viral aggregation as a result of interlinking particles or alternatively by binding bivalently preventing uncoating [18–20].

We have isolated two different human HPeV1 monoclonal antibodies (mAb) (Westerhuis *et al.*, submitted), of which AM18 was shown to be a broadly cross-neutralizing mAb against HPeV1, 2, 4, 5 and 6, and AM28 neutralized HPeV1 and HPeV2. These results indicated two different neutralizing epitopes for AM18 and AM28. Here, we present the epitopes identified for AM18 and AM28 and also their neutralization mechanism. The location of the virus neutralization epitopes on the capsid surface was revealed by peptide scanning for AM18. For AM28, we generated homology models of the HPeV1 capsid and the AM28 antibody as there are no atomic models available, and used these to interpret data from electron cryo-microscopy (cryoEM) and three-dimensional (3D) image reconstruction of AM28 complexed with HPeV1.

## Results

### Peptide scanning

To determine the specific binding region of the mAbs AM18 and AM28, overlapping 12mer peptides designed to cover the P1 (VP0, VP3 and VP1) sequence of HPeV1 were used in a peptide ELISA (biotin was bound to the N-terminus of the peptide without a linker) (Figure 1). The AM18 antibody showed strong binding to one peptide containing the RGD motif (peptide number 85; VTSSRALRGDMA), binding to a lesser extent to the second peptide containing the RGD motif (peptide number 86; ALRGDMANLTNQ), no binding to the preceding peptide (number 84: FFFPLPAPKVTs), and low binding to a peptide in the VP0 region (YGQsRYFAAVRC). This difference in binding between the two 'RGD' containing peptides indicates that the residues VTSSR N-terminal to the RGD increase the binding specificity, most probably by increasing the accessibility of the RGD motif due to the position of the RGD in the peptide (as no flexible linker was used between the biotin and the peptide of interest), not due to sequence specificity as peptide 84 showed no binding. The AM28 antibody showed no binding to the linear overlapping peptides in the ELISA (Figure 1), strongly suggesting that the epitope recognized by it is a non-linear, conformational-dependent epitope, hence we progressed with three-dimensional epitope mapping on the intact virions.

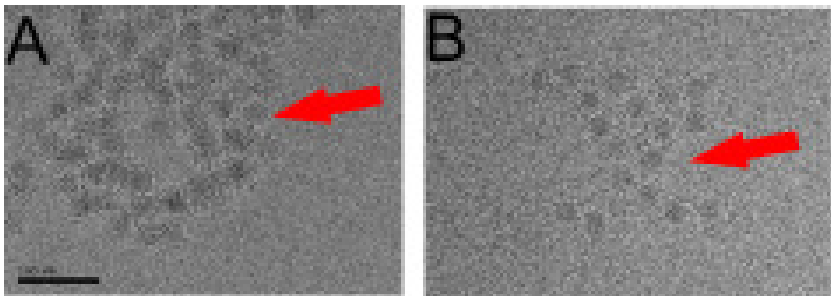


**Figure 1.** Peptide-scanning ELISA of AM18 and AM28. Plot of peptide number versus absorbance. The x-axis shows the peptides (P1 region) used to test binding with AM18 (A) or with AM28 (B) and the y-axis shows the absorbance at 540 nm after background subtraction from absorbance at 620 nm. Peptide number 85 (VTSSRALRGDMA) showed significant binding to AM18 compared to peptide number 86 (ALRGDMANLTNQ) even though both the peptides contain the 'RGD' motif.

## Virus-antibody complex

In order to understand the neutralization mechanisms and the exact epitopes of the mAbs AM18 and AM28, we imaged HPeV1 virions complexed with either mAb in a transmission electron microscope under cryogenic conditions. The micrographs did not show any noticeable disruption of HPeV1 virions in the presence of the mAbs antibodies AM18 or AM28, but they did induce strong aggregation of the HPeV1 virions (Figure 2).

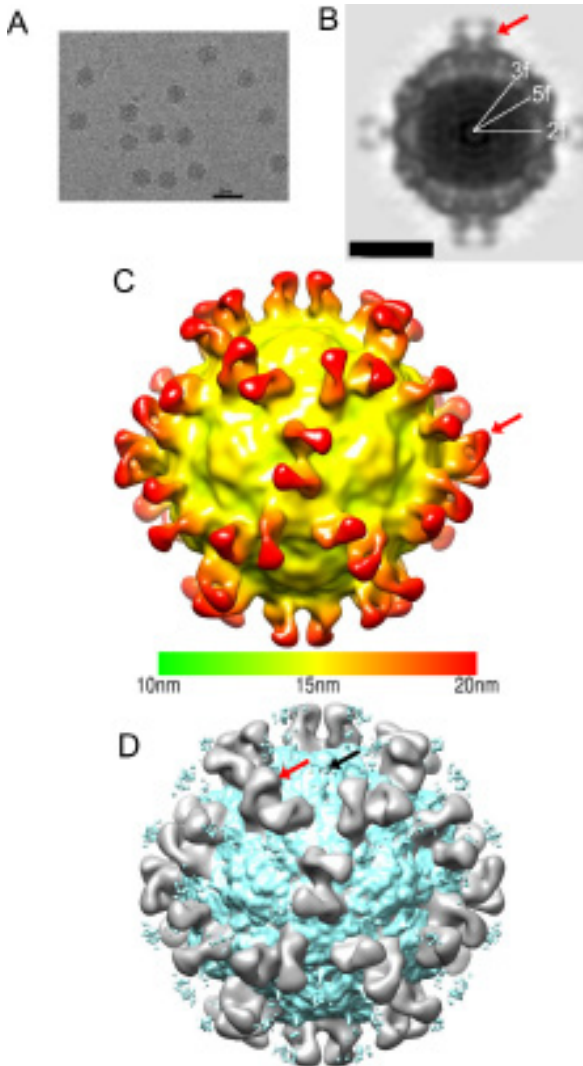
Since no linear epitopes were determined for AM28 using peptide scanning, we used cryoEM, 3D reconstruction, modelling and fitting to determine the binding site of AM28 on HPeV1. Due to aggregation of the virus in the presence of the mAb, we prepared AM28 Fab-labelled virus. The reconstruction statistics of HPeV1-AM28 Fab are summarized in Table 1 and the reconstruction is shown in Figure 3. In comparison with the virus reconstruction alone, the HPeV1-AM28 Fab reconstruction showed clear additional density attributable to the Fab density either side of the two-folds, bridging neighbouring pentamers (Figure 3B-C). This Fab footprint is distinct from the integrin footprint encompassing the VP1 RGD epitope that we have shown previously [6] (Figure 3D).



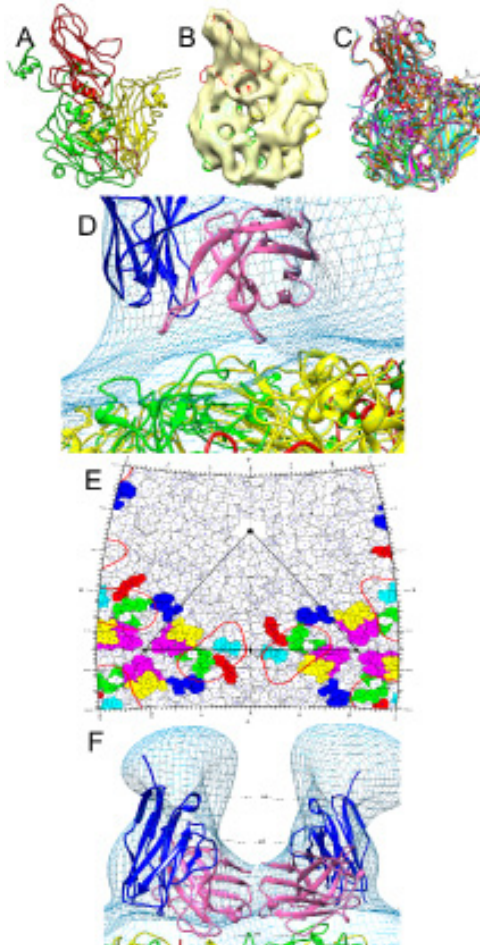
**Figure 2.** AM18 and AM28 antibodies recognise virus capsids leading to aggregation. Raw micrographs showing the aggregation (red arrow) of HPeV1 when mixed with AM18 Ab (A) or AM28 (B) at a molar ratio of 1(capsid):5 (antibody). (A & B) Scale bar in (A) is 100 nm.

**Table 1.** Statistics of the reconstruction

Parameter	HPeV1-AM28 Fab reconstruction
No. of micrographs	65
No. of particles used in the reconstruction	270
Underfocus range	1.65-4.06
Resolution (Å)	19.76



**Figure 3.** HPeV1-AM28 Fab reconstruction. A) Raw micrograph of HPeV1 in complex with AM28 Fab. Bar, 50 nm. B) Central cross-section of HPeV1-AM28 Fab complex with two-fold (2f), five-fold (5f) and three-fold (3f) symmetry axes marked. Scalebar 15 nm. C) Three-dimensional radially depth-cued reconstruction of the HPEV1 capsid with 60 Fab molecules bound. The reconstruction is colored according to the distance from the center of the particle. The color key is shown below the reconstruction. D) Overlay of the integrin-bound form of HPEV1 (EMD- 1689; greenish blue) with the HPEV1-AM28 (grey) complex showing different binding sites for integrin and antibody. A red arrow indicates one of the Fab molecules on the capsid surface and a black arrow indicates one of the positions where the integrin is bound.



**Figure 4.** Epitopes on HPeV1 for AM28. A) Homology models of VP1 (red), VP0 (yellow) and VP3 (green) built using I-TASSER. B) Final fits of VP1, VP0 and VP3 homology models into an asymmetric unit of HPeV1 (EMD-1690). C) Superimposing asymmetric units of echovirus 1 (PDB ID: 1EV1; magenta), poliovirus 1 (PDB ID: 1POV; grey), enterovirus 71 (PDB ID: 3VBF; orange) and foot mouth disease virus (PDB ID: 1QQP; cyan) on final fits of HPeV1 VP1 (red), VP0 (yellow) and VP3 (green). D) Mapping epitopes for AM28 on HPeV1 surface by fitting AM28 Fab variable region homology model into the Fab density seen in HPeV1-AM28 Fab reconstruction and superimposing VP1 (red), VP0 (yellow) and VP3 (green) fits for HPeV1 (EMD- 1690) into the HPeV1-AM28-Fab reconstruction (mesh). AM28 variable heavy chain is shown in magenta and variable light chain is shown in blue. E) Roadmap showing the density of AM28 Fab (red line contour, radius 155-156Å) and the epitopes HEWTPSWA (VP0; yellow), HQDKP (VP0; cyan), PLSIPTGSANQVD (VP0; magenta), MADSTTPSENHG (VP3; blue), ATTAPQSIVH (VP3; green) and FFPNATTDST (VP3; red). An asymmetric unit is marked by black lines. F) Distance between the symmetry-related Fab shown as wire.

In order to approximate the AM28 binding site, a homology model of the HPeV1 capsid was generated (Figure 4A) and subsequently superposed on the 20Å resolution HPeV1-AM28 Fab reconstruction. The highest confidence model was obtained for VP3 with an I-TASSER based confidence score (C-score) of -0.38 followed by VP0 with a C-score of -1.60. In contrast, the VP1 had a C-score of only -3.77. The typically C-score ranges from -5 to 2 with high score means better confidence in the quality of modelling [21]. In general, a C-score of -1.5 means more than 90% of the quality predictions are correct, thus, the VP1 model was only used to constrain the fitting of VP0 and VP3 in the asymmetric unit (Figure 4B-C). All the models had the characteristic eight-stranded  $\beta$ -barrels found in all picornaviruses capsid proteins (Figure 4C). Since the termini in picornaviruses are least conserved in the 3D conformation within the capsids and prediction was unreliable, we truncated the termini of the homology models. The placement of the individual capsid proteins within the capsid shell was improved using flexible fitting, resulting in improved fitting of the  $\beta$ -barrels and long helices of the models [22–24]. In addition a model of the AM28 Fab variable region was also generated and fitted into the Fab density in the HPeV1-AM28 Fab reconstruction. This showed that the antibody recognizes a conformational epitope which has contributions from both VP0 and VP3 (Figure 4D).

**Table 2.** Mapping conformational epitopes for AM28 and linear epitopes from peptide scanning of sera [13] on the capsid protein amino acid sequence.

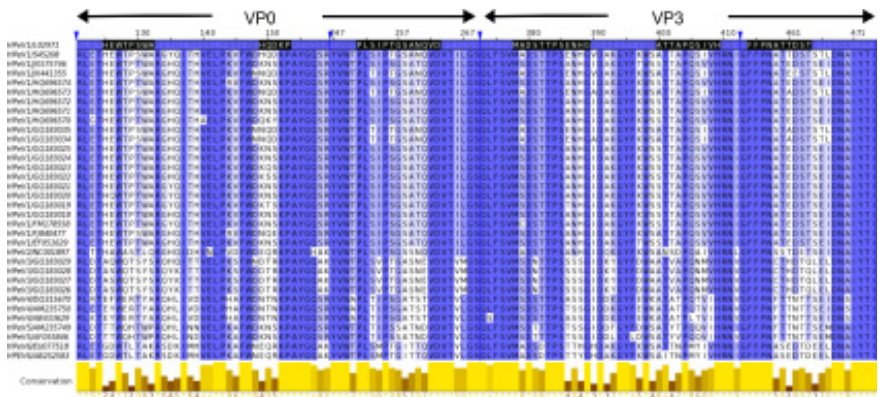
Capsid protein	Amino acid sequence*
VP0 (Genbank id: L02971, amino acids 1-289)	METIKSIADMATGVVSSVDSTINAVNEKVESVGNIEIGNLLTKVADDASNILGPN CFATTAEPENKNVQATTTVNTT <b>NLTQHPSAPTMPFSPDFSNV</b> DNFHSMAYDIT TGDKNPSKLVRL <b>ETHWTPSW</b> ARGYQITHVELPKVFW <b>DHQDKP</b> AYGQSRIFA AVRCGFHFQVQVNVNQGTAGSALVVYEPKPVVYD <b>SKLEFGAFTN</b> LPHVLMNL AETTQADLCIPYVADTNYVKT <b>DSSDLGQLKVVVWTP</b> <b>PLSIPTGSANQVDVITL</b> <b>GLSL</b> <b>LQLDFQNPRVFAQDVNIYDN</b>
VP3 (Genbank id: L02971, amino acids 290-542)	<b>APNGKKNWKKIMT</b> MSTKYKWTRTKIDIA <b>EGPGSM</b> NMANVLC <b>TTGAQSVALVG</b> ERAFYDPRTAGSKSRFD <b>DLVKIAQLFSV</b> <b>MADSTTPSENHG</b> VDAKGYFK <b>WSATT</b> <b>APQSI</b> VHRNIVYLR <b>LPNLNVFVNSYSYFRGSLVRL</b> LSVYAST <b>FN</b> RGR <b>LRMGFFP</b> <b>NATTDST</b> STLDNAIY <b>TICDIGSDNSFEITIPYSF</b> ST <b>W</b> MRK <b>TNGHPIGLFQI</b> EVLNRL TYNSSSPSEVY <b>Y</b> IV <b>Q</b> KG <b>M</b> Q <b>DARFFCPTGS</b> V <b>VT</b> F <b>Q</b>

\*Green: Antigenic sites identified by peptide scanning in [13]. Red: Antigenic sites for AM28 identified by fitting pseudo-atomic model fitting in EM density of HPeV1-Fab AM2. The loops in consecutive order are  $\beta$ B- $\beta$ C (VP0),  $\alpha$ A- $\beta$ D (VP0),  $\beta$ 1- $\beta$ H (VP0),  $\alpha$ Z- $\beta$ B (VP3),  $\beta$ B- $\beta$ C (VP3) and  $\beta$ E- $\alpha$ B (VP3).

Modelling and fitting of the HPeV1 VP0, VP1 and VP3 proteins indicated that amino acids in the following loops in HPeV1 were involved in the footprint:  $\beta$ B- $\beta$ C (VP0),  $\alpha$ A- $\beta$ D (VP0),  $\beta$ I- $\beta$ H (VP0),  $\alpha$ Z- $\beta$ B (VP3),  $\beta$ B- $\beta$ C (VP3) and  $\beta$ E- $\alpha$ B (VP3) (Figure 4D and E and summarized in Table 2 in red). These identified antigenic regions are distinct from linear epitopes of VP0 and VP3 that have been described previously by peptide scanning (Table 2, amino acids indicated in green) [16]. The fitting of the Fab variable region was unambiguous, with a cross correlation value of 0.88 compared to the 0.84 if the molecule was rotated by 180°. The distance between the two Fab molecules across the two-fold symmetry axis was on average about  $\sim 53\text{\AA}$  (Figure 4F).

### Conservation of the conformational epitope recognised by AM28

We compared amino acid sequence alignments of the newly identified VP0 and VP3 antigenic regions from different HPeV1 isolates with those of HPeV2-6. They were well conserved in HPeV1, moderately conserved in HPeV2 and poorly conserved in HPeV3-6 (Figure 5) which explains why mAb AM28 cross-binds and cross-neutralizes HPeV2, but no reactivity was detected against HPeV3-6 (Westerhuis *et al.*, submitted).

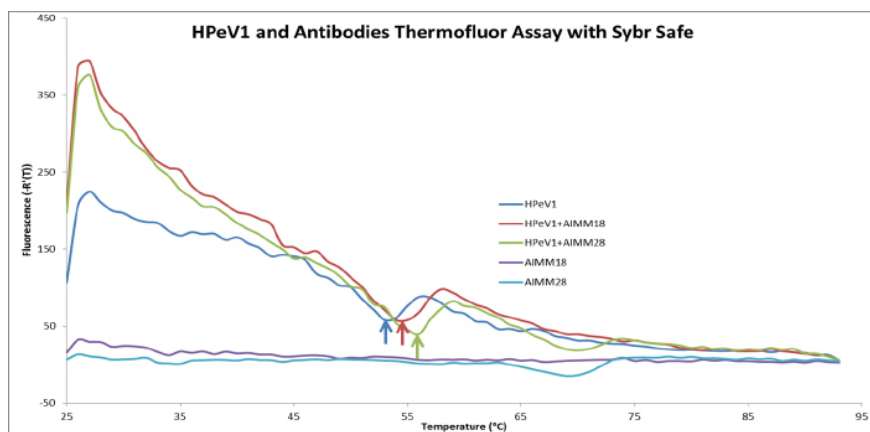


**Figure 5.** Conservation of epitopes. Amino acid sequences of HPeV1-5 used for neutralization in Westerhuis *et al.*, (submitted) were aligned against complete genome sequences for HPeV1-6. The sequence annotation on the left hand side is 'virus genotype/GenBank ID'. The epitopes are marked in black on the HPeV1-Harris strain (GenBank ID: L02971) that was used as the basis for the HPeV1 homology modelling. The alignment is coloured according to percent sequence identity, from a scale of white (no identity) to dark blue (full identity). The conservation panel in yellow below the alignment gives the numerical values for the conservation based on the BLOSUM 62 score of the alignment and physicochemical conservation where \* is 100% identity. The blue arrowheads indicate irrelevant regions of the sequence that have been hidden in the final representation for simplicity (1-120, 160-246, 270-372, 413-453). The figure was made with Jalview.



## Mode of neutralization

In order to understand the mechanism of neutralization, we performed a capsid thermal stability assay using an RNA binding dye, which has previously been used to explain the mode of action of EV71 neutralizing antibodies [19]. The  $T_m$  of the HPeV1, HPeV1-AM18 and HPeV1-AM28 were 53°C, 54°C and 56°C respectively (Figure 6). The 3°C shift in  $T_m$  for HPeV1-AM28 compared to HPeV1 alone indicates that the bivalent binding of AM28 across neighbouring pentamers stabilizes the capsid, inhibiting RNA release from the capsid. In contrast, a shift of 1°C in  $T_m$  for HPeV1-AM18 suggests that increasing the capsid stability is not the primary reason for neutralization by AM18.



**Figure 6.** Thermofluor assay. Plot of temperature (x-axis) versus first derivative of fluorescence (y-axis) showing the change in  $T_m$  when HPeV1 is bound to antibodies AM18 or AM28 compared to HPeV1 alone. Arrows indicate the  $T_m$  for each sample. AM18 and AM28 were used as the negative control for the RNA binding dye.

## Discussion

HPeV infections can be severe and even life threatening, especially for neonates. Severe infections in neonates can be due to a lack of protective maternal Abs. No treatment is currently available against these severe HPeV infections, making it an unmet medical need [25]. For HPeVs, Ab-based therapies are a feasible option as the HPeV genus is a relatively small group of highly similar viruses for which cross-reactive, neutralizing polyclonal Abs have already been described [16,17]. For a successful antiviral approach, knowledge of specific and overlapping viral antigenic sites is important and mAbs are preferred. We developed two different mAbs AM18 and AM28 which were able to neutralize HPeV1 efficiently at IC<sub>50</sub>s of 2.6 to 5.5 ng/ml (Westerhuis *et al.*, submitted). These mAb were cross-reactive with HPeV2 and AM18 also showed cross-reactivity against HPeV4. Peptide-scanning ELISA analysis confirmed that the site of the AM18 binding was within the sequence 'ALRGDMA' as this was the common sequence between two positive overlapping peptides from the VP1 C-terminus. Of this, "RGDMA" is also common to HPeV4 VP1 and "RGD" is common to HPeV2 VP1; it is missing from HPeV3. Hence, the minimally-recognised epitope for neutralization is "RGD" and AM18 most likely neutralises by directly competing with the cellular receptor (integrins) for this binding site, diminishing the ability to infect cells through this route. AM18 recognises and neutralizes other RGD containing viruses like coxsackievirus A9 (Westerhuis *et. al.* submitted), but it did not neutralize Echoviruses presumably because they can use alternative receptors to enter the host cells. For HPeV1 it has been shown that RGD-less mutants with reduced binding to integrin are less viable [12], it may therefore be difficult for the virus to generate escape mutants resistant to AM18 neutralization. Hence AM18 shows potential as a useful therapeutic molecule.

AM28 did not recognize linear epitopes from denatured proteins or overlapping peptides in Western blot or ELISA, indicating that the epitopes are conformational. We used homology models of the VP0, VP1, VP3 and AM28 fitted into a HPeV1-AM28 Fab reconstruction to identify a conformational epitope which has contributions from VP0 and VP3 of neighbouring pentamers. Although we have little experimental data to show the validity of the homology models, as there are no atomic models for parechoviruses that we are aware of, the position of the RGD epitope in VP1 agrees to within 5 Å with the position of the integrin footprint found earlier [6] and the fit of the obvious elements of secondary structure, such as the β-barrels and the VP0 helices forming the interface at the two-fold are consistent with what is expected from the literature. Comparison of known picornavirus structures with that of our capsid model indicates that this conformational epitope is a region commonly occupied by the equivalent loops from VP2 and VP3, defined by the conserved position of the β-barrels. Hence, even though we could not trace the chain of

the capsid proteins, or identify amino acid side chains, we are confident in the prediction of the loops contributing to the footprint. The sequence conservation between HPeV1 and HPeV2 supports this prediction, as both are neutralized by AM28. From the reconstruction AM28 appears to staple neighbouring pentamers together thereby stabilizing the whole capsid. This hypothesis was tested by measuring the fluorescence of an RNA binding dye when the virus capsid was heated in a step-wise manner from 25°C to 95°C. The idea was that upon heating, the capsid would destabilize at a certain temperature, thus allowing the dye increased access to the RNA. AM28 had a stabilising effect on the capsid. Thus the  $T_m$  was increased by 3°C compared to the virus alone. Significantly, breathing of picornavirus capsids for RNA release has been shown to be dependent on domain movements that open up the interfaces at the two-folds [26–32]. Hence, the monovalent binding of one Fab arm of AM28 across the two-folds, would prevent this movement, stabilize the capsid, prevent uncoating of RNA from the capsid on cell entry, and thus neutralize the virus. The avidity of AM28 would be higher because both the Fab arms can bind the neighbouring epitopes as the distance between the two Fab arms is about 53Å when bound to the capsid [20,33]. Interestingly, this conformational epitope did not overlap with any of the linear immunogenic epitopes identified previously by peptide scanning [16] indicating that such single dimensional epitope mapping techniques may miss some of the crucial epitopes on the capsid surface which are only presented in the tertiary form. The importance of this area of the capsid in capsid assembly and RNA delivery is shown in the conservation of these loops in multiple HPeV1 and HPeV2 isolates, but not in the other serotypes. Hence this mAb also shows therapeutic promise for HPeV1 and HPeV2 infections offering a glimmer of hope to the many patients that are afflicted. The atomic model of HPEV1 will be of use in understanding mutations in the capsid that affect the tropism of the virus – an area of great interest in understanding the transmission from the respiratory and gastrointestinal tract to the central nervous system.

## Material and Methods

### Virus culture and purification

HPeV1-Harris was provided by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands and grown on a human colon carcinoma cell line (HT29). The HT29 cells were maintained in Eagle's Minimum Essential Medium (EMEM) with L-glutamic acid (0.2X), non-essential amino acid (1X), streptomycin (0.1 µg/ml) and ampicillin (0.1 µg/ml), supplemented with 8% heat-inactivated fetal calf serum (FCS). The virus concentration was determined by the median tissue culture infective dose (TCID<sub>50</sub>) and calculated by the Reed and Muench method [34]. For the large scale

virus purification 90% confluent cell layers in T-175 flasks were infected with HPeV1 at a multiplicity of infection 0.1. After 75-100% infection of the cell monolayer evident by the cytopathic effect, the cells and spent media were freeze-thawed twice at  $-80^{\circ}\text{C}/+37^{\circ}\text{C}$ , centrifuged at 4000 rpm for 15 minutes in an Eppendorf A-4-62 swing bucket rotor at  $4^{\circ}\text{C}$  and the supernatant was filtered using a  $0.22\ \mu\text{m}$  filter. The virus was pelleted by ultracentrifugation at 32000 rpm for 2 h at  $4^{\circ}\text{C}$  in a Beckman SW32Ti rotor. The pellet was dissolved in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM  $\text{MgCl}_2$  (1X TNM buffer) and loaded on to a cesium chloride step gradient with a 5ml 40% (w/v) bottom layer and a 5ml 15% (w/v) top layer and centrifuged at 32000 rpm, for 16 h at  $4^{\circ}\text{C}$  in a Beckman SW41Ti rotor. The fraction containing the virus was concentrated with a 100kDa cutoff filter (Millipore) in 1X TNM.

### **Antibody preparation and virus labeling**

The human AM18 and AM28 antibodies were prepared as described previously (Westerhuis *et al.*, submitted). In brief, human memory IgG+ B cells were cultured using the AIMSelect method [35,36] and antibody-containing culture supernatants were used to directly screen for HPeV1 neutralization. Fab fragments from AM28 were produced using a Pierce Fab micro preparation kit according to the manufacturer's protocol. The resulting Fab was mixed with HPEV1 capsids at a molar ratio of 5:1 in 1x TNM buffer for 30 minutes at room temperature.

### **Peptide-scanning enzyme linked immunosorbent assay**

Streptavidin-coated ELISA plates (Greiner bio-one) were blocked with 2% BSA in PBS for 2 h at room temperature. N-terminally biotin-labelled HPeV1 overlapping peptides (12aa in length with 6aa overlap and no flexible linker) (Antonie van Leeuwenhoek, Netherlands cancer institute, Peptide Synthesis) were diluted (1:500) in 1% BSA in PBS and bound to the plate for 1 h at room temperature. The plate was incubated with  $2\ \mu\text{g}/\text{ml}$  of AM18 or AM28 for 1 h at room temperature and washed 3 times with PBS/0.1% Tween. Anti-human IgG HRP-labeled ( $0.3\ \mu\text{g}/\text{ml}$ ) was used as the secondary Ab, incubated for 1 h at room temperature and washed three times with PBS/0.1% Tween. The substrate solution containing 3,3',5,5'-tetramethylbenzidine was added and incubated for 10 min at room temperature in the dark. The reaction was stopped by the addition of 0.8 M  $\text{H}_2\text{SO}_4$ . The absorbance at 450 nm and 620 nm was measured with a microplate reader.

## Electron cryo-microscopy

Aliquots of the Fab-virus mixture were vitrified on Quantifoil R2/2 holey carbon nickel grids in a home-built guillotine by plunging into liquid ethane maintained in a liquid nitrogen bath. After vitrification, the grids were stored in liquid nitrogen until use. The grids were examined in a FEI F20 transmission electron microscope at 200 keV using a Gatan 626 cryostage. The images were recorded on a Gatan Ultrascan 4000 under low dose conditions at a magnification of 69000X with a sampling size of 2.17 Å per pixel.

The contrast transfer function of each micrograph was estimated using CTFFIND3 and images containing drift or astigmatism were discarded [27]. Particles were picked using the program ETHAN [37] with a box size of 401 pixels and inspected by eye in the program suite EMAN [38]. A previous reconstruction of HPeV1 from Seitsonen *et al.*, 2010 (EMD-1690) was used as a starting model to initiate full orientation and origin determinations of the Fab labelled set of images using AUTO3DEM ver4.03.1 [39]. The final reconstructions calculated to the Nyquist frequency were used to estimate the B-factors with EM-Bfactor, and then the reconstructions were truncated to the resolution indicated by the Fourier shell correlation analysis with a threshold criterion of 0.5 [40,41]. The HPeV1-AM28 Fab density map was deposited in the Protein Databank in Europe (accession number EMD-XXXX).

## Homology modelling and fitting of models into cryoEM maps

The structures of the three HPeV1 capsid proteins were predicted by multiple-template comparative modeling using the I-TASSER server [30]. The template structures for VP0 were foot and mouth disease virus (PDB ID: 1QQP, 1FMD and 1BBT) [42,43], poliovirus 1 (PDB ID: 1POV) [44], bovine enterovirus (PDB ID: 1BEV) [45] and Seneca Valley virus-001 (PDB ID: 3CJI) [46]. For VP1, they were triatoma virus (PDB ID: 3NAP) [47], human rhinovirus 14 (PDB ID: 1D3I) [48], cricket paralysis virus (PDB ID: 1B35) [49], rabbit hemorrhagic disease virus (PDB ID: 4EJR) [50], echovirus 7 (PDB ID: 1M11) [51] and bovine enterovirus (PDB ID: 1BEV) [45]. For VP3, they were human enterovirus 71 (PDB ID: 3VBF) [28], Seneca Valley virus-001 (PDB ID: 3CJI) [46], human rhinovirus 16 (PDB ID: 1AYM) [52], poliovirus Mahoney strain (PDB ID: 1HXS) [53].

An atomic model of echovirus 1 capsid (PDB ID: 1EV1) [54] was placed into an 8.5 Å resolution HPeV1 map (EMD-1690) [6]. The fitting was done using a modification of a protocol described elsewhere [24]. The homology models were aligned with the echovirus 1 capsid, before being rigidly fitted into the HPeV1 map (EMD-1690) [6] using the 'fit in map' feature in UCSF-Chimera [55]. The N-terminus of VP0, VP3 and VP1 were truncated to avoid inter-subunit clashes. Using the 'zoning' feature in UCSF-Chimera [55], the HPeV1

capsid map was zoned to an asymmetric unit with a radius of 6 Å using the truncated VP0-VP3-VP1 rigidly-fitted model. RIBFIND based rigid bodies were identified for the truncated VP0-VP3-VP1 model [24] and the model was flexibly fitted into the asymmetric unit using one iteration in FlexEM [22] followed by iMODfit based flexible fitting using the default settings [23]. The resulting homology model of the complete HPeV1 capsid was then placed directly into the Fab-labelled reconstruction to identify the probable binding sites. The variable regions of AM28 Fab were modelled using the WAM webserver [56] and manually fitted into the corresponding Fab density in the HPeV1-AM28 Fab reconstruction and the fit was optimized by 'fit in map' feature in UCSF-Chimera. All the visualization was carried out in UCSF-Chimera [55]. The fitted models are deposited in the Protein Databank in Europe with the PDB ID: XXXX.

### Sequence alignment

The P1 amino acid sequences of HPeV1 (GenBank ID: L02971, GQ183023, GQ183022, GQ183021, GQ183020, GQ183019, GQ183018, GQ183025, GQ183024), HPeV2 (GenBank ID: NC\_001897), HPeV3 (GenBank ID: GQ183026), HPeV4 (GenBank ID: DQ315670) and HPeV5 (GenBank ID: AF055846) used for AM18 and AM28 neutralization assays (Westerhuis *et al.*, submitted) were aligned using Clustal Omega [57] with additional HPeV strains for which the complete genome sequences were available in GenBank (GenBank IDs for HPeV1 are JX441355, JX575746, S45208, EF051629, FJ840477, GQ183035, GQ183034, HQ696574, HQ696572, HQ696570, HQ696573, HQ696571, FM178558; for HPeV3 are GQ183027, GQ183028, GQ183029; for HPeV4 are AB433629, AM235750; for HPeV5 is AM235749; for HPeV6 are EU077518, AB252583). The alignment was visualized with Jalview [58].

### Thermofluor assay

In order to test the capsid stability in the presence of antibodies, AM18 and AM28 mAb were mixed with HPeV1 virions (so capsids containing RNA) at a molar ratio of 66:1 and incubated at room temperature for 30 min. Dye-accessibility to the RNA increasing with heat was detected with a fluorescent dye. The reaction volumes were set up per well in a 96-well PCR plate and each reaction contained 2.5 µl of 200X Sybr Safe DNA gel stain (Invitrogen, also binds RNA) and the protein sample which was one of the following HPeV1 (10 µl of 1mg/ml stock), AM18 (10 µl of 2 mg/ml stock), AM28 (10 µl of 2 mg/ml stock), HPeV1-AM18 complex (20 µl) or HPeV1-AM28 complex (20 µl). The total volume was made up to 25 µl for each reaction volume using 1X TNM buffer. The assay was run from 25°C to 95°C with readout every 0.33 s in an Mx3005P qPCR instrument (Agilent Technologies). The Sybr Safe DNA gel stain dye was excited at 492 nm and emission was read at 516 nm.

## Acknowledgements

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# 8.

## **Polyclonal and monoclonal Abs against HPeV3 lack the ability for efficient neutralization of HPeV3 viral infection.**

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## Abstract

Within the genus of *Parechovirus*, human parechovirus (HPeV) genotype 3 has been shown to differ in various aspects from HPeV1, 2, 4, 5 and 6. In contrast to infection with most of the HPeV genotypes, HPeV3 infection has frequently been reported to cause severe disease in infants. Since absence of neutralizing antibodies in serum against closely related enteroviruses has been shown to be a risk factor for severe disease in infants, prophylactic and possible therapeutic treatment of human picornavirus infections with mAbs is a rational option. In this study we generated a polyclonal rabbit IgG by rabbit immunization with HPeV3 and three different HPeV3 specific human monoclonal Abs. These human mAbs were generated by screening supernatants of antibody-producing B cell cultures, derived from donors who experienced HPeV3 infection, for direct binding to HPeV3 infected cells by fluorescent microscopy. In contrast to the polyclonal HPeV rabbit IgG that inhibited HPeV3 infection to some extent and which showed cross-binding with all HPeV1-6 genotypes, the three human mAbs AT12-015, AT12-017 and AT12-018 were specific for HPeV3 binding only, but were not able to inhibit HPeV3 replication *in vitro*.

## Introduction

Within the genus *Parechovirus*, human parechovirus (HPeV) genotype 3 has been shown to differ in various aspects from HPeV1, 2, 4, 5 and 6. HPeV3 was first isolated in 2004 from a stool specimen from a 1-year old child with transient paralysis (1). After this report more often severe cases of HPeV3 infection were reported, showing significantly more severe symptoms during infection with HPeV3 compared to infections with HPeV1 (1–6). Moreover, HPeV3 is the most predominant subtype detected in cerebrospinal fluid (CSF) (3, 7–11), showing the importance of HPeVs in CNS infection. Children infected with HPeV3 are younger (<6 months) compared to children infected with HPeV1 (>6 months), suggesting that maternal HPeV3 specific antibodies are absent or are non-protective and thereby the newborn is vulnerable to infection.

Although classified as a different Picornavirus genus, HPeVs are closely related to human enteroviruses (HEVs). During enteroviral infections humoral immunity is thought to play a major role in clearing the virus. Maternal Abs derived before birth and during breastfeeding may play an important role in protection of neonates against picornavirus infections. A previous study showed an association of high maternal antibody titers in serum and in breast milk with a reduction of the frequency of EV infections (12). Thereby, absence or low levels of neutralizing maternal-derived HEV specific antibodies, have been shown to be related to more severe infection in neonates (13). In case of HPeVs high levels of neutralizing Ab titers against HPeV1, 2, 4, 5 and 6 can be detected already at young age (14). Two Finnish studies showed anti-HPeV1 seroprevalence of >97% in adults, and of the 21 neonates tested, 95% had HPeV1 Abs, which suggested maternal Ab protection against HPeV1 (15, 16). This in contrast to data obtained in HPeV3 seroprevalence studies indicating that levels of neutralizing Abs against HPeV3 are generally low e.g. 4% in Finland and 8% in the Netherlands. Even more strikingly we only found 1 child out of 330 (age 0 to 5 yrs) positive for antibodies neutralizing HPeV3 (14). In addition we showed that in two adults with a history of HPeV3 infection, no neutralizing serum Abs could be detected during, nor one year after infection (17). In a Japanese study in adults it was shown that up to 71 days after onset of a HPeV3 infection, mostly very low neutralizing Ab titers were induced, indicating an inefficient Ab response against HPeV3 (18). The lack of neutralizing (maternal) Abs against HPeV3 is a major risk, since no treatment is available, although neonates with severe HEV infection occasionally receive intravenous immunoglobulins (IVIg) as supportive treatment. Whether this treatment is truly effective remains elusive since high titers are needed against the specific serotype to be beneficial against severe infection (13, 19). We showed in a previous study that, compared to the other genotypes, protective Abs against HPeV3 were also low (titer <1:40) in different IVIg batches (17).

The well-characterized arginine-glycine-aspartic acid (RGD) motif in the C-terminal of the VP1 capsid protein of HPeV1, shown to be important in receptor binding and Ab neutralization, is absent in HPeV3. We previously isolated two highly neutralizing mAbs against HPeV1 (AM18 and AM28), even showing cross-neutralization with HPeV2, 4, 5 and 6, but not with HPeV3 (Westerhuis *et al.*, 2014, submitted). Showing that next to the RGD motif, other epitopes in the VP0 and VP3 capsid proteins are involved in Ab neutralization (Shakeel *et al.*, submitted). The absent cross-binding and neutralization by AM18 and AM28 of HPeV3 suggest that the neutralizing epitope recognized by these antibodies is absent in HPeV3. The identification of specific HPeV3 neutralizing Abs would provide a good tool for further studies in HPeV3 Ab neutralization and receptor binding. Secondly, neutralizing specific HPeV3 mAbs would provide a good option in antiviral treatment of severe HPeV3 infection in neonates. In this paper we describe the characteristics of a polyclonal Ab and three novel human mAbs against HPeV3.

## Materials and Methods

### Virus culture and purification

HPeV1-Harris and the HPeV2-2008 strain were provided by the Dutch National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands, the primary strains HPeV3-150237, HPeV4-251176, HPeV5-552322 and HPeV6-550389 were obtained in house. Two additional HPeV3 strains, HPeV3-0252277 and HPeV3-1051930 were isolated from feces and passaged twice to obtain a sufficient viral stock. For virus culture of HPeV1, 2, 4, 5, and 6, the human colon carcinoma cell line HT29 was used, while HPeV3 was cultured on African green monkey kidney (Vero) cells. The cells were maintained in Eagle's Minimum Essential Medium (EMEM) with L-glutamic acid (0.2X), non-essential amino acid (1X), streptomycin (0.1 µg/ml) and ampicillin (0.1 µg/ml), supplemented with 8% heat-inactivated Fetal Calf Serum (FCS). The virus concentration was determined by the median tissue culture infective dose (TCID<sub>50</sub>) and calculated by the Reed and Muench method (20). Total virus purification was performed as described before (Westerhuis *et al.*, 2014, submitted)

### Rabbit polyclonal Ab preparation and Antibody discovery

Polyclonal Abs were obtained by rabbit immunization (Harlan Laboratories, United Kingdom). The purified virus was used for immunization of one rabbit. The rabbit received 5 times every 14 days a boost of 200µg purified virus and 0.5ml FCA/FIA adjuvant. The final bleed was taken after 77 days.

The AT12-015, AT12-017 and AT12-018 antibodies were obtained from healthy human donors generated as described previously (21, 22). In brief, CD27+ memory B cells were isolated from peripheral blood by FACS sorting. Cells were stimulated for 36 hrs with CD40L and interleukin (IL) 21 and subsequently transduced with a retrovirus containing the Bcl-6 and Bcl-xL transgenes together with the marker gene GFP. The presence of HPeV3 Abs in B cell supernatants was tested by binding to HPeV3 infected cells as shown by immunofluorescence. Cultures showing binding were single cell subcloned to obtain monoclonal B cell cultures. The antibody heavy and light chain genes were retrieved from these B cell clones and expressed as recombinant protein in 293T cells. IgG1 antibodies were subsequently purified using HiTrap Protein A columns on an ÄKTA instrument (GE).

### **Immunofluorescence assay**

The immuno-fluorescence was performed as described previously (17). The cells were incubated with the primary Abs, AT12-015, AT12-017 and AT12-018. As secondary Ab 15 µg/ml of anti-human IgG (Alexa 488) was used and the nucleus was stained with 0.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma).

### **Expression and purification of HPeV capsid proteins**

To generate recombinant capsid protein VP0, VP3 and VP1 from the HPeV1-Harris cDNA strain and the virus isolates HPeV2, 3, 4 primer sequences were used as depicted in Table S1, using the following PCR: 5' 95°C, followed by 35 cycles 30s at 95°C, 30s at 55°C and 60s at 72°C and an extension step of 10' at 72°C. The purified PCR fragments were cloned into the expression vector Pet102 with his-tag and expressed in *Escherichia coli* BL21 Star™(DE3) One Shot® cells. Single bacterial colony was inoculated in LB medium supplemented with 100 µg/ml ampicillin and propagated at 37°C with 220rpm speed on a shaker incubator till the culture reached logarithmic growth phase (at OD600 0.6–0.7). To stimulate recombinant protein expression 0.5mM isopropyl β-D-thiogalactopyranoside (IPTG) was added to the cultures. The proteins were purified by using Ni-NTA purification system with Anti-His(C-term)-HRP Ab in accordance with the manufacturer's instructions (K953-01, Invitrogen).



Table S1. Primers for generating the different capsid proteins

		Primer [5' – 3']	
		Forward	Reverse
HPeV1	VP0	CACCATGGAGACAATTAAGAGTATTGC	ATTATCATATATGTTGACATCTTGAGCA
	VP3	CACCGCACCAATGGTAAAAAG	TGGAATGTAACAACAGAACCA
	VP1	CACCAATTCATGGGGTTTCCAC	CTGATTTGTAAGGTTTGCCATATC
HPeV2	VP0	CACCATGGAGACAATCAAGAGCATTGCAG	TGTATCGTAAATGTCACATTTTGCCCATACACC
	VP3	CACCGCACCTCTAAACCAATCCATTGAG	CTGGAAAGTTACTAAGACCCAGTGGGGCAG
	VP1	CACCAATTCATGGGGTTCCCAAATGGAC	TTGGTCAGACCATGTTGCCAAATCTCCTC
HPeV3	VP0	CACCATGGAGTCTATCAAGGATTTAGTC	ATTATCATAAATTTCCACATCTTCACCA
	VP3	CACCGACCTAATAAGGCCAATGCTA	TTGGAAGGAGACCAGAGAACCAGT
	VP1	CACCAATTCATGGGGTTTCCAC	TTTATCATAAAGTCTGTCAGC
HPeV4	VP0	CACCATGGAGACAATCAAGGGTAT	TTTATCATAAAGTCTGTCAGC
	VP3	CACCAACAATGACACTAGGGC	TTGGAATGTAACAAGAGAGCCCT
	VP1	CACCAATTCATGGGGTTTCCAC	CTGGTCTGAGAAGTTTGCCAT

## ELISA

ELISA plates were coated with 200ng of purified HPeV1-6, or the different capsid proteins VP0, VP3, VP1 from HPEV1-4 overnight at room temperature (RT). After washing the plates were washed with 0,1%tween/PBS (3 times) the ELISA plates were blocked with 2% BSA in PBS for 2 hours at room temperature. The plate was incubated with 2 µg/ml of the HPeV3 Abs for 1 hr at RT and washed 3 times with PBS/0.1% Tween. Anti-human IgG HRP labeled (0.3 µg/ml) was used as the secondary Ab, incubated for 1 hr at RT and washed three times with PBS/0.1% Tween. The substrate solution containing 3,3',5,5'-tetramethylbenzidine was added and incubated for 10min at RT in the dark. The reaction was stopped by the addition of 0.8 M H<sub>2</sub>SO<sub>4</sub>. The O.D. at 450nm and 620nm was measured with a microplate reader.

## Virus neutralization assay

Abs of 0.25 mg/ml concentration were used for an endpoint neutralization assay, Abs (7.5µg/ml – 0.03µg/ml) were mixed with HPeV3 virus suspension containing 100 TCID<sub>50</sub>/50µl. Mixtures were incubated at 37°C for 1hr, and were used to inoculate different cell lines Vero, BGM (buffalo green monkey kidney), A549 (human colon adenocarcinoma) and Caco2 (human colon adenocarcinoma) cells (on a 96-wells plate (200 µl). As positive control cells were inoculated with 100TCID<sub>50</sub> without Abs present, as negative controls only Abs (1:16) or only medium was added to the cells. The cells were examined for the appearance of CPE every 24h for 7 days. At day 7, 25µl of supernatant was removed for RNA isolation using

the total nucleic acid isolation kit with the MagnaPure LC instrument® (Roche Diagnostics). The RNA was eluted in 50µl elution buffer and reverse transcribed as previously described. Five µl of cDNA was used for to estimate viral copy number using LC480® real time PCR machine (Roche Diagnostics). The virus copies per PCR were calculated with a standard curve as previously described (23, 24)

## Results

### Human monoclonal antibody generation

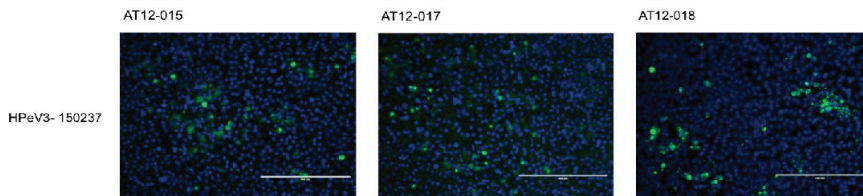
Human memory CD27+IgG+ B cells were obtained from peripheral blood from two healthy donors with a proven HPeV3 infection one year before donation (Wildenbeest *et al.*, in preparation). The B cells were cultured at different cell densities per well in 96 well plates, and the supernatants were tested for binding to HPeV3 infected cells. From the cultures showing binding to HPeV3 infected cells, single B cell cultures were generated to retrieve the original monoclonal B cell. The supernatants from the selected clones were also tested for direct neutralization of HPeV3 infection. All of the binding clones were selected and RNA was isolated to retrieve the antibody heavy and light chain sequences (Table 1), unique sequences were used to generate recombinant protein from 293T cells.

**Table 1.** Antibody sequences characteristics

Clone	Isotype	IGHV	IGHD (frame)	IGHJ	CDR3H	aa replacement
						VH
AT12-015	IgG1	3-23*01	3-16*01	4*02	CAKRLGRVAEYFFDYW	7
AT12-017	IgG1	3-48*03	6-19*01	6*02	CARILLGQQWLPTYYYYGMDVW	10
AT12-018	IgG1	3-23*01	5-12*01	4*02	CAKRLGRVAEYFFDYW	7 (1 aa del in FR2)
		IGKV/LV	IGK/LJ		CDR3L	VL
AT12-015	κ	1-05*03	4*01		CQQYNNYMALTF	8
AT12-017	κ	1-05*03	2*04		CQEYNNYPMCSF	3
AT12-018	κ	1-05*03	4*01		CQQYNTYFALTF	5

### Antibody binding to HPeV3 infected cells

The rabbit polyclonal aHPeV3-HARLAN Ab and the three different purified mAbs AT12-015, AT12-017 and AT12-018 were tested for binding to HPeV1-6 infected cells by immunofluorescence. The polyclonal HPeV3 rabbit IgG showed cross-binding to all 6 genotypes (data not shown), while the three mAbs were specific in HPeV3 binding (Figure 1). To identify the Ab binding to the different capsid proteins, the Abs were tested in an ELISA for binding to whole purified virus, and different recombinant denatured capsid proteins VP0, VP3 and VP1. The polyclonal HPeV rabbit IgG showed binding to whole virus and the capsid proteins VP0 and VP3 of the genotypes HPeV1, 2, 3 and 4. AT12-015, -17 and 18 showed specific binding to purified HPeV3 only, and not against the different capsid proteins. These data were confirmed with western blot (data not shown).



**Figure 1.** Detection of AT12-015, AT12-017 and AT12-018 binding to HPeV3 infected cells by Immuno-fluorescence staining. Vero cells were used for infection with HPeV3. When CPE was observed the cells were fixed with 4% paraformaldehyde and stained with the monoclonal Abs. Antibody binding was detected with a secondary goat anti human IgG- Alexa-488 labeled (Green). Nuclei were detected using DAPI.

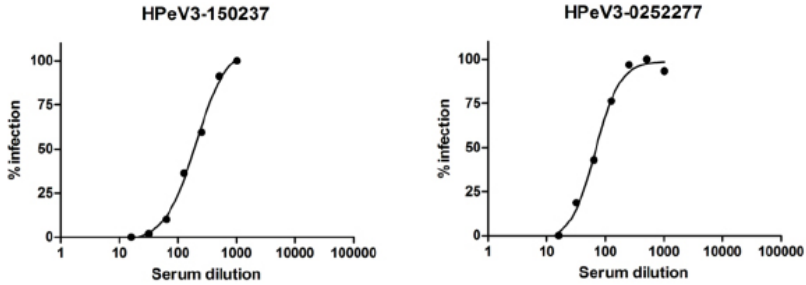
**Table 2.** Binding of HPeV3 polyclonal Ab and monoclonal Abs to purified HPeV1-6 and the different capsid proteins in an ELISA

	Strain		aHPeV3-HARLAN	aHPeV3- AT12-015, AT12-017 and AT12- 018 Ab
Purified virus	HPeV1-Harris		+	-
	HPeV1B		+	-
	HPeV2		+	-
	HPeV3		+	+
	HPeV4		+	-
	HPeV5		+	-
	HPeV6		+	-
Capsid proteins	HPeV1	VP0	+	-
		VP3	-	-
		VP1	+	-
	HPeV2	VP0	+	-
		VP3	-	-
		VP1	+	-
	HPeV3	VP0	+	-
		VP3	-	-
		VP1	+	-
	HPeV4	VP0	+	-
		VP3	-	-
		VP1	+	-

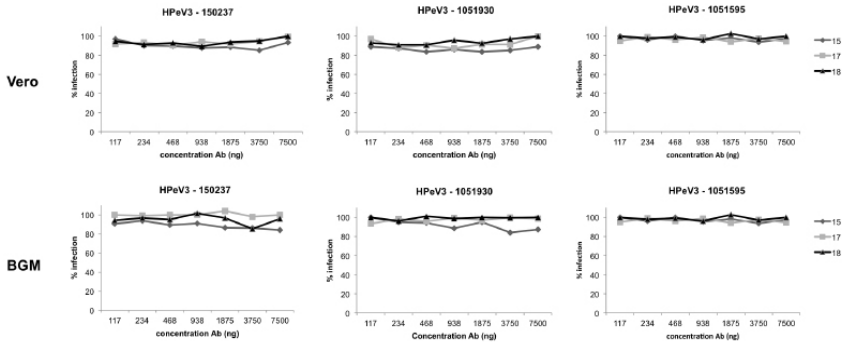
### Antibody neutralization of HPeV3

The HPeV3-specific polyclonal and monoclonal Abs were tested for neutralization capacity against 100TCID<sub>50</sub> of HPeV1-6, by monitoring CPE as well as the decrease in viral copy numbers in real time PCR (Figure 2, 3). The polyclonal HPeV rabbit IgG was able to neutralize HPeV3-150237 at an IC<sub>50</sub> dilution factor of 205 and HPeV3-0252277 at an IC<sub>50</sub> dilution factor of 68. Although AT12-015, -17 and 18 did recognize HPeV3 infected cells the mAbs could not neutralize HPeV3 infection at 10 or 100TCID<sub>50</sub> on Vero cells. To exclude any influence of cell tropism, we also tested neutralization of different HPeV3 clinical isolates on the BGM cell line (Figure 3). At most AT12-015 Ab was able to inhibit viral replication of our prototype HPeV3-150237 strain with a maximum of 1 log viral copies in both the Vero and BGM cell lines. The highest inhibition that could be observed was a 15%

(2 log) difference in replication of the HPeV3-1051930 strain in the BGM cell line at high Ab titers (Figure 3). The AT12-017 and AT12-018 did not inhibit viral infection of any of the HPeV3 strains. Moreover, the Abs did not neutralize HPeV1, 2, 4, 5 or 6 (data not shown).



**Figure 2.** Neutralization of HPeV3- 150237 and HPeV3- 0252277 using the polyclonal HPeV rabbit IgG. An end point neutralization assay is performed with 100TCID<sub>50</sub> of HPeV3. The viral copies per sample are measured by PCR at day 7 at the different serum dilutions.



**Figure 3.** Neutralization of primary HPeV3- 150237, - 0252277 and - 1051930 strains using the three different mAbs AT12-015, AT12-017 and AT12-018 in a) Vero and b) BGM cells. An end point neutralization assay is performed with 100TCID<sub>50</sub> of HPeV3. The viral copies per sample are measured by PCR at day 7 at the different AB concentrations.

## Discussion

In this study we generated a polyclonal HPeV rabbit IgG by total HPeV3 antigen immunization, and three human monoclonal Abs specific for HPeV3 from donors who experienced HPeV3 infection one year before blood was obtained. The polyclonal HPeV rabbit IgG generated against HPeV3-150237 did show specific neutralization but only at very low dilution. This is in agreement with what we observed for the Japanese Ab (A308-99), which was able to specifically neutralize the HPeV3-A308-99 strain at very low dilutions (1:10), but was not able to neutralize the HPeV3-150237 strain, despite efficient binding to infected cells (Westerhuis *et al.*, 2013). Thus two independently generated polyclonal rabbit Ab sera against HPeV3, the polyclonal HPeV rabbit IgG and aHPeV3-A308 Ab (Japan), were not able to neutralize HPeV3 infection efficiently at low dilutions, in contrast to what is shown for polyclonal rabbit sera generated against HPeV1. To study the human monoclonal antibody response against HPeV3, we developed 3 novel IgG1 antibodies specific for HPeV3. All three mAbs specifically recognized HPeV3 infected cells, they did not neutralize HPeV3 infection *in vitro*. Previously, we generated mAbs against HPeV1 using the same B cell immortalization technique but then we could directly screen for neutralization, something that was technically impossible for HPeV3. Using the direct neutralization screening assay it proved to be relatively easy to obtain mAbs with high neutralization titer against HPeV1 (Westerhuis *et al.*, 2014 submitted). In general HPeV3 neutralization *in vitro* is more difficult and possibly requires higher antibody concentrations. Therefore, the screening and selection for HPeV3 specific mAbs in our study was based on specific binding which resulted in HPeV3 specific antibodies that however did not neutralize in the standard HPeV3 *in vitro* neutralization assays. Interestingly, the polyclonal Ab generated by immunization showed cross-binding to HPeV1-6, but specific albeit low neutralization against HPeV3. Thus it could be that by selecting HPeV3-specific binding Abs, neutralizing Abs were missed or that more than one antibody in the rabbit sera synergistically neutralized the virus. For the generation of HPeV3 specific mAbs, initially all positive supernatants were tested for HPeV3 neutralization, but no neutralizing Ab could be selected. It could be that potential neutralizing Abs were not present in concentrations high enough to obtain neutralization. A second possibility, which is supported by the lack of high neutralization of the polyclonal Abs, is that both aHPeV3 polyclonal and monoclonal Abs are not able to efficiently reach the neutralizing epitope and cannot disturb the binding to the (unknown) receptor, and thus infection. Since HPeV3 neutralization by human sera is also absent or weak (14, 25, 26), this supports our previous suggested hypothesis that maternal Ab protection might fail even when Abs are present, a risk of severe disease in neonates. This together may suggest that the general humoral immune response to HPeV3 is weak and not very potent, and therefore other defence mechanism (T-cell or innate immunity) are responsible for clearing HPeV3 infection.

Another hypothesis is that the Abs are not able to neutralize viral infection in *in vitro* experiments. For FMDV it has been shown that mAbs passively protect neonatal syngeneic (BALB/c) mice at dilutions which they could not neutralize virus infection *in vitro* (27), suggesting that opsonisation and phagocytosis play a major role in the immune response. To test this hypothesis animal models are needed. It has been shown that the availability for animal models for HPeV infections is limited. Newborn mice inoculated with HPeV1 and HPeV2 were only infrequently infected, and cynomolgus monkeys showed no neuropathological changes after 30 days of observation (28).

In this study we were not able to isolate highly neutralizing mAbs against HPeV3. Secondly we could not identify the location of the specific mAb binding epitope of HPeV3 among the three different capsid proteins. This indicates that the mAbs bind a conformational epitope of the virus particle. Knowledge of the specific HPeV3 epitopes would also be of great value in the development of a specific HPeV3 assay, for example an ELISA specifically detecting HPeV3 Abs in sera, as the detection of (neutralizing) Abs in serum appeared to be difficult. For the identification of these epitopes and to study the differences in neutralization and receptor binding between HPeV1 and HPeV3 further research is needed. One approach is cryo- electron microscopy to reveal the HPeV3 structural features and to identify the specific binding epitopes of the non-neutralizing HPeV3 mAbs described in this study.

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# 9.

## **Anatomy of a human parechovirus 3 at near-atomic resolution**

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## Abstract

Human Parechovirus 3 (HPeV3) is one of the main causative agents of meningitis and sepsis in neonates. Currently, there are no therapies available to combat its infection, but insight into its structure could help design of antiviral therapies. This virus is unique among the *Parechovirus* genus as it lacks the 'arginine-glycine-aspartic acid' motif in the C-terminus of the capsid protein VP1, used by the others to gain entry into cells using integrins. Here, we present a high-resolution structure of HPeV3 as determined by cryo-electron microscopy and image reconstruction. The HPeV3 structure has features similar to enteroviruses such as raised 5-fold and 3-fold vertices, an open channel through the capsid and an annulus below each 5-fold vertex. By fitting homology models of HPeV3 into the EM density we have identified loops, which could be utilized as epitopes for generation of therapeutic antibodies against the HPeV3 capsid. Overall, the HPeV3 structure gives the first instance of high-resolution structural information for the *Parechovirus* genus.

## Introduction

The *Picornaviridae* family contains both a large group of enteroviruses (EVs) as well as a smaller group of parechoviruses (HPEVs) that are prevalent causes of viral infections in humans. HPEV infections are mainly mild, causing respiratory symptoms and gastrointestinal symptoms, but can also result in sepsis-like disease and CNS symptoms. HPEV genotype 3 is one of the main causative agents of meningitis and sepsis-like illness in neonates. Despite the importance of HPEV3 in CNS involvement no antiviral therapies are available to combat HPEV3 infections. HPEV3 was first isolated in 2004 from a stool specimen from a 1-year old child with transient paralysis (1). Since then, several studies have highlighted significantly different clinical and biological differences between HPEV3 and the other prevalent genotypes HPEV1, 2, 4, 5 and 6. Clinically, HPEV3 is most often associated with severe disease (1–6).

The genome of HPEVs consists of a ~7.5kb, single-stranded, positive-sense RNA, which is enclosed in a capsid containing 60 copies of each capsid protein (VP0, VP3 and VP1). HPEV3 lacks the arginine-glycine-aspartic acid (RGD) motif in the VP1 C-terminus VP1, which has been shown to be essential in viability and infection of HPEV1. HPEV1 utilises via the RGD motif the  $\alpha v\beta 3$ ,  $\alpha v\beta 6$  integrins as their receptors (7–10), for entry into the host cell by a clathrin-dependent endocytic pathway (8). Structural studies on HPEV1 confirmed the importance of the RGD motif and their interaction with the  $\alpha v\beta 3$ ,  $\alpha v\beta 6$  integrins (10). The missing RGD motif in HPEV3 implies different receptor use. Therefore, we generated three different HPEV3 mAbs which showed specific binding, but failed to neutralize HPEV3 replication *in vitro* (Manuscript in preparation). These mAbs most likely have non-linear epitopes. Further studies in the differences in receptor binding and neutralization are essential in understanding viral infection and pathogenesis, and in the design of antiviral therapies. Towards this end, the 3D reconstruction of HPEV3 from cryo-EM could be of significant benefit. In this study we generated present a high-resolution structure of HPEV3 as determined by cryo-electron microscopy, giving the first instance of high-resolution structural information for the *Parechovirus* genus.

## Materials and Methods

### Virus culture, purification and inactivation

HPeV3 isolate 152037 was and grown on the African green monkey, Vero cell line. The Vero cells were maintained in growth medium composed of Dulbecos Modified Eagles Medium (DMEM) with glutamax (1X), non-essential amino acid (1X), streptomycin (0.1 µg/ml), penicillin (0.1 µg/ml) and 10% heat-inactivated fetal bovine serum (FBS). A 90% confluent cell layers were inoculated with HPeV3 at a multiplicity of infection (MOI) of 0.01. At 100% infection of the cell monolayer evident by the cytopathic effect (CPE), the cells and spent media were collected and freeze-thawed thrice at -70°C and 37°C, centrifuged at 4000 rpm for 15 minutes in an Eppendorf A-4-62 swing bucket rotor at 4°C and the supernatant was filtered using a 0.22 µm filter. The virus was concentrated using 100kD cutoff membrane filters (Millipore). The concentrate was pellet was loaded on to a cesium chloride step gradient with a 5ml 40% (w/v) bottom layer and a 5ml 15% (w/v) top layer and centrifuged at 32000 rpm, for 16 h at 4 °C in a Beckman SW41Ti rotor. The virus band was buffer exchanged with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl<sub>2</sub> (1X TNM buffer) using a 100kD cutoff membrane filter (Millipore). The purified HPeV3 was inactivated by adding 0.1 mg/ml formaldehyde and keeping at 37°C for 72h. Inactivation was confirmed by infecting Vero cells with inactivated virus. No cytopathic effect was seen up to 7 days.

### Electron cryo-microscopy

Aliquots of the purified HPeV3 were vitrified on Quantifoil R3.5/1 holey carbon nickel grids in a home-built guillotine by plunging into liquid ethane maintained in a liquid nitrogen bath. After vitrification, the grids were stored in liquid nitrogen until use. The grids were examined in a Cs corrected FEI Titan Krios transmission electron microscope at 300 keV. The images were recorded on a back-thinned Falcon II detector under low dose conditions at a nominal magnification of 59000X with a sampling size of 1.14 Å per pixel. Seven frames per image were collected in counting mode using FEI EPU automated single particles acquisition software. The integrated unaligned image was used for further image processing. The contrast transfer function of each micrograph was estimated using CTFFIND3 and images containing drift or astigmatism were discarded (11). Particles were picked using the program ETHAN (12) with a box size of 401 pixels and inspected by eye in the program suite EMAN (13). Random model generated from 150 particles (EMD-1690) was used as a starting model to initiate full orientation and origin determinations of the of full set of particles using AUTO3DEM ver4.04.1 (14). The final reconstructions calculated to the Nyquist frequency were used to estimate the B-factors with EM-Bfactor, and then the reconstructions were truncated to the resolution indicated by the Fourier shell correlation analysis with a threshold criterion of 0.5 (15, 16). The HPeV3 density map will be deposited

in the Protein Databank in Europe.

### **Homology modelling and fitting of models into cryoEM maps**

The structures of the three HPeV3 capsid proteins were predicted by multiple-template comparative modeling using the I-TASSER server (17). The template structures for VP0 were foot and mouth disease virus (PDB ID: 1QQP and 1BBT) (18, 19), poliovirus 1 (PDB ID: 1POV) (20), bovine enterovirus (PDB ID: 1BEV) (21), Theiler's murine encephalomyelitis virus (PDB ID: 1TME) (22), equine rhinitis A virus (PDB ID: 2WFF) (23) and Seneca Valley virus-001 (PDB ID: 3CJI) (24). For VP1, they were human enterovirus 71 (PDB ID: 3VBF) (25), triatoma virus (PDB ID: 3NAP) (26), human rhinovirus 14 (PDB ID: 1D3I and PDB ID: 1NCQ) (27, 28), human rhinovirus 16 (PDB ID: 1AYM) (29), echovirus 1 (PDB ID: 1EV1) (30), and staphylococcal enterotoxin H (PDB ID: 1EWC) (31). For VP3, they were poliovirus 1 (PDB ID: 1POV) (20), coxsackievirus A9 (PDB ID: 1D4M) (32), equine rhinitis A virus (PDB ID: 2XBO) (33), poliovirus Mahoney strain (PDB ID: 1AL2) (34), echovirus 1 (PDB ID: 1EV1) (30), Seneca Valley virus-001 (PDB ID: 3CJI) (24) and bovine enterovirus (PDB ID: 1BEV) (21). An atomic model of echovirus 1 capsid (PDB ID: 1EV1) (30) was placed into a 5.45 Å resolution HPeV3 map. The fitting was done using a modification of a protocol described elsewhere (35). The homology models were aligned with the echovirus 1 capsid, before being rigidly fitted into the HPeV3 map using the 'fit in map' feature in UCSF-Chimera (36). Using the 'zoning' feature in UCSF-Chimera (36), the HPeV3 capsid map was zoned to an asymmetric unit with a radius of 6 Å using the VP0-VP3-VP1 rigidly-fitted model. Residues 1-65 of VP0, 1-70 of VP3 and 1-36 of VP1 were truncated because these were the regions of lowest confidence in the homology models. The truncated VP0-VP3-VP1 model was flexibly fitted into the asymmetric unit using iMODfit with the default settings (37). All the visualization was carried out in UCSF-Chimera (36). The fitted model will be deposited in the Protein Databank in Europe.



## Results

### HPeV3 structure

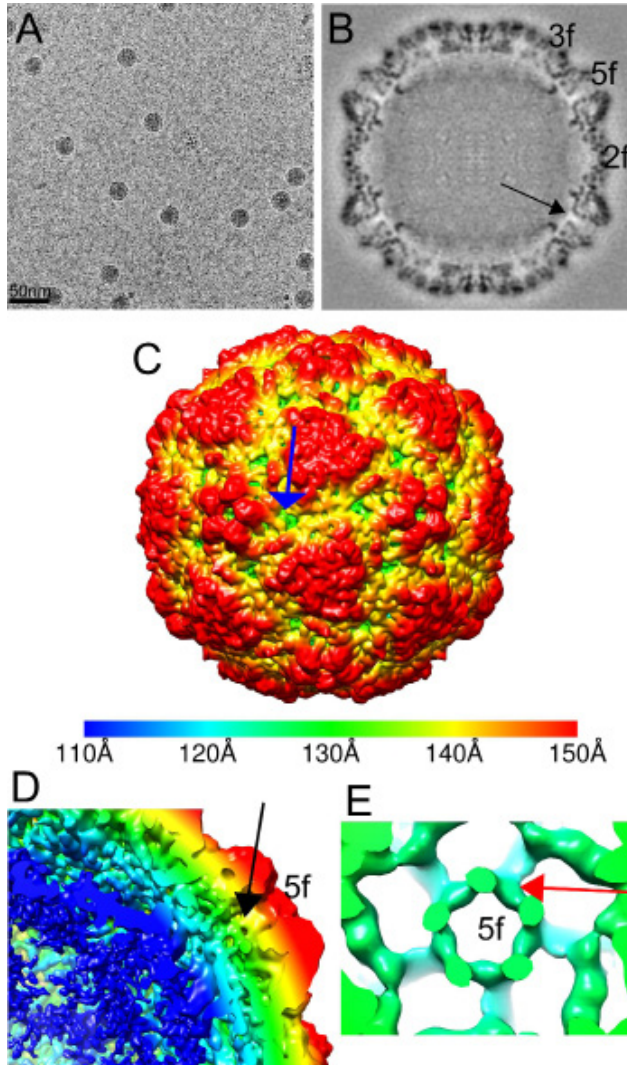
Using a stable microscope Titan Krios with a back-thinned direct electron detector, we were able to reach a resolution of 5.45 Å with as few particles as 1276 (Figure 1A-C and Table 1). The micrographs had a high signal to noise ratio, which greatly aided in determining the orientation and origin centres correctly. Using a random model generated initially from 150 particles as a reference model, the full dataset converged within 207 iterations. The HPeV3 structure shows features similar to other picornavirus such as the presence of a canyon on the capsid surface and the presence of an annulus below the five-fold vertices (Figure 1 C and D). Similar to CVA7, the HPeV3 five-fold vertices also have an open channel which stays open even when viewed at a threshold close to the mean density of the map (38).

**Table 1:** Statistics of the reconstruction.

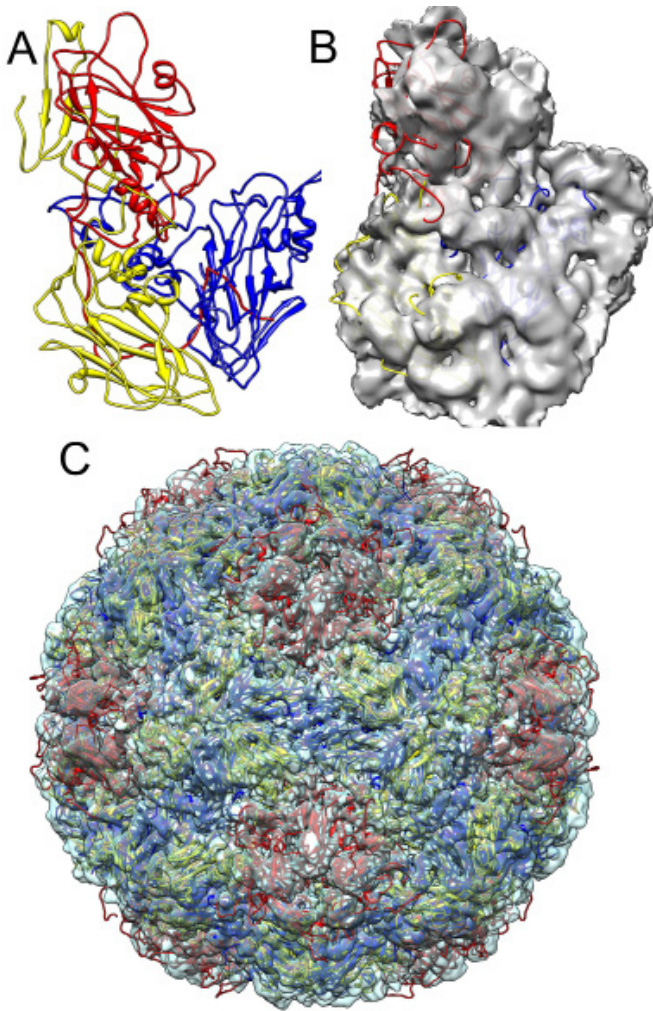
Parameter	HPeV3 reconstruction
No. of micrographs	237
No. of particles used in the reconstruction	1276
Underfocus range	0.42-2.34
Resolution (Å) based on 0.5 FSC	5.45
B-factor applied	50

### Pseudo-atomic model of HPeV3

The I-TASSER (17) based homology model of VP0 had a model confidence score (C-score) of -0.21, VP3 had a C-score of -0.48 and VP1 had the lowest C-score among the three capsid proteins of -2.06 (Figure 2A). Due to homology modelling errors, the N-termini of the capsid proteins were truncated before being flexible fitted into the HPeV3 asymmetric density. The  $\beta$ -barrels, helices and loops constrained by secondary structure elements fitted well into the EM density whereas long loops and the termini fitted with low confidence (Figure 2B). The pseudo-atomic model generated by applying symmetry operators on the VP0, VP3 and VP1 asymmetric fit agreed well with the HPeV3 capsid density, although there were a few clashes especially between neighbouring asymmetric units (Figure 2C).



**Figure 1.** HPeV3 structure. A) Raw micrograph showing the HPeV3 capsids at defocus of 2  $\mu\text{m}$ . Scale bar 50 nm. B) Cross-section view of HPeV3 with five-fold (5f), three-fold (3f) and two-fold (2f) symmetry axes marked. C) Radially depth-cued isosurface representation of HPeV3 at resolution of 5.45 Å with a bfactor of 50. D) Central-cross section of HPeV3 showing the open channel at five-fold vertex. E) A slab of thickness 30 Å showing an annulus below the five-fold symmetry vertex. The black arrow shows the open channel at five-fold vertices in B and D, the blue arrow shows the canyon on the HPeV3 surface in C and the red arrow shows the annulus.



**Figure 2.** Pseudo-atomic model of HPeV3. A) Homology models of HPeV3 capsid protein from I-TASSER. B) Fitting of homology models into an asymmetric unit of HPeV3 EM density. C) Pseudo-atomic model of HPeV3 full capsid. Model of VP1 is shown in red, VP0 in blue and VP3 in yellow. EM density is shown in a UCSF Chimera surface representation with transparency.

## Discussion

HPeV3 are highly pathogenic in neonates, and the great differences shown compared to the well studied HPeV1 makes it essential to investigate HPeV3- structural properties for better understanding of HPeV3 pathogenesis. Here we determined a 5.45Å resolution reconstruction of HPeV3 showing features similar to other picornaviruses. Noticeable, is the open channel in the five-fold axis, which was not seen in the reconstruction of HPeV1, but has been frequently observed in other picornaviruses such as enterovirus CAV7 (38). As well as the two-fold channel, in case of CAV16 the conformational changes during uncoating results in an increase of the cross section area of the two fold axis channel (39). The channels in other picornaviruses, both on the five-folds and the two-folds have been implicated in the conformational changes in all the capsid proteins that are initiated during receptor binding, release of pocket factors, emergence of VP4 and the termini of VP1 that are required for RNA genome release. HPeVs do not contain the VP4 capsid protein per se, it remains as the uncleaved N-terminus of VP0, and the mechanism of viral uncoating remains unclear. Additional experiments are needed to look into the mechanism of viral uncoating and the role of the conformational changes in HPeV3 during RNA release.

Further analysis of this pseudo-atomic model of HPeV3 could be utilized to identify surface exposed residues on the capsid and comparison with other known picornaviral structures is ongoing. Thereby further comparative sequencing, I-TASSER, MODELLER, Robetta, and refined alignment techniques can lead to the prediction of the receptor binding site of HPeV3, as shown for HRVC, where modelling had led to insights in receptor preference and immunogenicity (40).

The HPeV3 structure can be used for the prediction and design of mAb targets in the fight against HPeV infection. Secondly the reconstruction of the HPeV3 labelled with the non-neutralizing mAbs is ongoing. This analysis will be of great value in the understanding of the HPeV3 genotype and the development in antivirals.

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# 10.

## Summary and General Discussion



## Summary

HPeVs are widespread pathogens, infecting mainly young children worldwide. Transmission of HPeVs seems to occur via the fecal-oral route and most HPeV infections are mild, associated with respiratory tract infection and gastro-enteritis. HPeV infections are linked to a variety of severe diseases such as sepsis-like illness, myocarditis, encephalitis, meningitis and paralysis, most pronouncedly caused by the genotype HPeV3. Ever since HPeV3 was described for the first time in 2004, several reports showed association of HPeV3 with severe disease, and HPeV3 being the predominant type detected in CSF, showing the importance of HPeVs in CNS infections. Additionally children infected with HPeV3 are significantly younger than HPeV1 infected children. Not only with respect to clinical features but also with respect to biological features HPeV3 differs from the other genotypes known. HPeV3 is more difficult to propagate in cell culture, and HPeV3 lacks the arginine-glycine-aspartic acid (RGD) motif, which has been shown to be critical for integrin binding of HPeV1, and is an important antigenic site of HPeV1. In this thesis the differences shown between HPeV3 and the other genotypes is further investigated.

### HPeV cell tropism

Several studies showed difficulties with HPeV detection in cell culture, because of only a low induction of cytopathic effect (CPE). In **Chapter 2** we showed the different replication kinetics of the culturable HPeV genotypes 1 to 6 on a set of commonly used cell lines measuring viral copies/pcr with real time PCR. Replication monitored by PCR showed that growth of HPeV genotypes 1 to 6 was supported by most of the cell lines tested. By comparing replication kinetics, measured by PCR, and the CPE, we showed that viral replication could be measured before CPE appeared in the infected cell line, while sometimes CPE did not occur at all. In three of the nine cell lines (Vero, RD99 and A549) all six prototypes could be propagated. All HPeV1 to 6 genotypes showed replication with high viral titers on the RD99 cell line, but CPE was hardly seen. The combination of HT29 and Vero cells is suitable to detect all culturable HPeV types by CPE, where viral replication could be detected within 3 days. HPeV3 was shown to replicate on all cell lines except HT29.

For several EV genotypes, differences in disease manifestations and severity have been related to differences in cell tropism. In **Chapter 3** we explored this for HPeVs by comparing the replication kinetics of HPeV1 and HPeV3 strains isolated from patients with known clinical symptoms in different continuous cells lines. Using real time PCR to detect replication, the HPeV3 strains showed better replication kinetics on the neural cell line SH-SY-5Y. Virus strains isolated from 3 out of 4 patients with CNS involvement showed better replication on the neural cell line SH-SY-5Y, reaching up to 3-fold higher viral titers,

than viruses from patients without CNS involvement. However this association could not be shown for the HPeV1 strains isolated from patients with severe symptoms. Additionally we could not link the strains isolated from patients with mild respiratory or gastrointestinal symptoms to replication in the lung carcinoma (A549) or in the colon carcinoma cell lines (HT29 and Caco-2). All the cell lines used in the previous studies are continuously growing cell lines obtained from tumours that do not necessarily represent the tissue from which they originated. In **Chapter 4** the Human Airway Epithelial cells (HAE) was introduced as a primary cell culture system for HPeVs. After infection with the different HPeV1-6 genotypes no recognizable CPE was visible in the HAE cells. Efficient replication was shown for HPeV1, 2 and 3, while the replication of HPeV4, 5 and 6 was very low or absent. HAE cells isolated from different donors were infected with HPeV1 and 3 and in four out of five donors efficient viral replication could be shown, with faster replication of HPeV3. For the first time we were able to propagate two of the more recently discovered HPeVs, namely HPeV9 and HPeV14 which both lack the RGD motif. Therefore the HAE cell culture system provides a good tool to further study tropism and pathogenesis of the different HPeVs, including the previously unculturable RGD-less HPeVs.

### **HPeV Ab neutralization**

We hypothesized that the age difference between children infected with HPeV1 and HPeV3 could be due to lack of the maternal Ab protection against HPeV3 in the first 6 months of life. High seroprevalence have been described for HPeV1 and 2, however, seroprevalence data on HPeV3 in the adult population are only available from Japan. Therefore we conducted a seroprevalence study in **Chapter 5**. Here we described the nAb positivity against HPeV1-6 genotypes in 554 sera obtained from Finnish and Dutch individuals. High nAb positivity was found for HPeV1 and HPeV2 (86%-99%) in the total adult study population in both countries. The high nAb positivity against HPeV2 (86% and 95%) is difficult to comprehend because HPeV2 viruses have been detected very rarely over the last decades in Finland and the Netherlands. We speculate that the high HPeV2 seropositivity could indicate HPeV cross-neutralization and thus cross-protection among the different genotypes. Chapter 5 also described the first seroprevalence data on HPeV4-6. Seroprevalence of HPeV4 was similar in both populations (60% in Finland and 62% in the Netherlands) whereas the seroprevalence of HPeV5 (75% vs. 35%;  $p < 0.001$ ) and HPeV6 (74% vs. 57%;  $p = 0.04$ ) was significantly higher in the Dutch adult population compared to the Finnish, which is in line with the prevalence of the viruses found in these countries. As expected the Ab positivity increases with age; for HPeV1 more than half of the children with age up to 5 years were already HPeV1 nAb positive. In contrast, we observed a very low nAb positivity against HPeV3, 4% in Finland and 8% in the Netherlands. This is seemingly in discordance with the high frequency of HPeV3 detection in both countries. One explanation could be a

very recent introduction of HPeV3 in the population. However, in **Chapter 2** we already showed that the polyclonal Ab against HPeV3 we obtained from Japan failed as well to neutralize HPeV3 infection *in vitro*, while neutralization of HPeV1, 2, 4, 5 was efficient with their representative polyclonal Ab. And in **Chapter 3**, IVIg (IgGs from >1000 donors) manufactured between 2005-2010 was shown to contain high neutralizing Ab titers against HPeV1, but hardly neutralized HPeV3 strains. Moreover, very low neutralizing Ab titers were found in serum of two HPeV3-infected donors at time of infection as well as one year post infection, while high neutralizing Ab titers against HPeV1 could be detected in these donors.

To further investigate virus neutralization, in **Chapter 6** we generated a polyclonal rabbit IgG mixture by rabbit immunization with HPeV1, as well as two HPeV1 specific monoclonal Abs, AM18 and AM28. The mAbs were generated by screening culture supernatants of antibody producing B cell cultures obtained from adult donors for direct neutralization of HPeV1. Both polyclonal and monoclonal Abs showed specific HPeV1 neutralization, but also neutralization of HPeV2. In addition, HPeV1 specific mAb AM18 showed cross-neutralization against HPeV4, 5 and 6, and even against the HEV CAV9. This Ab cross-reactivity seems to be restricted to strains containing the RGD motif at the C-terminus of the capsid protein VP1. A VP1 specific ELISA confirmed that the AM18 indeed bound the capsid protein of HPeV1, 2 and 4. In contrast, the HPeV1 specific mAb AM28, which neutralized HPeV1 much more efficiently than AM18, showed no cross-reactivity with the other HPeVs or HEVs and did not react with any of the capsid proteins, suggesting a non-linear epitope for this Ab. To identify the non-linear epitope for mAb AM28, Cryo-EM studies were performed on HPeV1 viral particles, labelled with AM28-Fabs (**Chapter 7**). We showed that the AM28 Fab indeed did not overlap with the integrin binding site, which is the binding site for AM18. The antibody footprint was found to be across two protomers from adjacent pentamers in the capsid recognizing both VP0 and VP3. The conformation recognized by AM28 requires two adjacent protomers from two different pentamers, one contributing VP0, the other contributing VP3. Three-dimensional reconstruction and pseudo-atomic model fitting that AM28 recognizes quaternary epitopes on the capsid composed of VP0 and VP3 loops from neighbouring pentamers. Reconstruction suggests that the AM28 Ab staples the neighbouring pentamers together thereby stabilizing the whole capsid, preventing uncoating. In **Chapter 8** the generation and characterization of aHPeV3 Abs is described. A rabbit polyclonal aHPeV3-HARLAN Ab and the three different purified mAbs AM15, AM17 and AM18 were generated with the same techniques as in **Chapter 7**. The B cell cultures for generation of HPeV3 mAbs were isolated from healthy donors, who experienced a HPeV3 infection in the past, and specificity for HPeV3 was tested by capacity to bind HPeV3. The polyclonal Ab was able to bind all 6 genotypes, but

specifically neutralized HPeV3 although at very high concentrations of Abs. The mAbs all showed specific binding to HPeV3, but showed no binding to the different capsid proteins, indicating a non-linear epitope. None of the three mAbs could neutralize HPeV3. Structural studies can give good insights in to the differences in receptor usage and Ab binding of HPeV1 and HPeV3. This will lead us to a better understanding of the pathogenic difference between type 3 and other HPeV. With cryo-EM we resolved a high resolution of HPeV3 shown in **Chapter 9**. The HPeV3 structure has features similar to enteroviruses such as the presence of canyon around the 5-fold vertices and annulus below the vertices. Different from HPeV1, the HPeV3 viral particle has an open channel at the 5-fold vertices. By fitting homology models of HPeV3 into the EM density we have identified loops, which could be utilized as epitopes to generate therapeutic antibodies against the HPeV3 capsid.

## General discussion

### Human Parechovirus infection: bowel or brain, that's the question?

HPeVs have been shown to be important pathogens for humans throughout the world. HPeV1 is the most frequently detected genotype of all EVs and HPeVs in stool samples, and seem to be more often associated with mild gastro-intestinal symptoms compared to EVs. HPeV3 was even identified as the most common picornavirus type detected in CSF in the UK (1). The transmission route of HPeVs is supposed to be fecal-oral, and the main site of replication for HPeVs is thought to be the gut. The route to CNS infection remains unknown. Two essential factors in invasion of the CNS, and thus severe disease, are cell tropism and the immune response against HPeVs. Previous studies showed that HPeV3 differs from the other genotypes and is significantly more associated with severe symptoms (2–8). HPeV3 was thought to be only clinical relevant in infants, whereas more recent reports showed symptomatic HPeV3 outbreaks among adults in Japan (9). This knowledge indicates a great need for antiviral treatment, as there is no treatment against HPeVs available at the moment. In our studies we show the importance of cell tropism and Ab protection in the pathogenesis of HPeV infection.

### HPeV cell tropism, implications for pathogenesis

The differences in pathogenesis between HPeV3 and the other genotypes could be explained by different receptor use and thus different cell tropism. For HPeV1 it has been shown that it utilizes the vibronectin  $\alpha\beta 3$  and  $\alpha\beta 6$  integrins as its receptors, via the RGD motif (10–12). The receptor and the receptor binding motif for HPeV3 remains unknown. Given the lack of the RGD motif in HPeV3, it is likely that HPeV3 uses a different receptor than HPeV1. Comparing the different growth characteristics of HPeV1-6 and different HPeV1 and HPeV3 strains isolated from patients with known clinical outcome, we could not show a clear relation between pathogenicity of HPeVs in different standard cell lines tested. We showed that 3 out of 4 virus strains isolated from patients with CNS involvement showed better replication on the neural cell line SH-SY-5Y, reaching up to 3-fold higher viral titres, than viruses from patients without CNS involvement. But for HPeV1 a correlation between *in vitro* replication dynamics and disease severity could not be found (Chapter 3). Additionally we could not link the strains isolated from patients with mild symptoms to replication in the lung carcinoma (A549) nor in the colon carcinoma cell lines (HT29 and Caco-2). For CAV9 and E9 a relationship between clinical manifestations and growth characteristics *in vitro* has been suggested, which could even be linked to the presence of the RGD motif. For CAV9 and E9 it has been shown in mice, that lack of the RGD motif was related to a lower pathogenicity (13–15). However, not all E9 strains isolated from patients with paralysis were able to cause paralysis in mice, showing the same inconsistencies as in

our studies. (14). This link between higher pathogenicity and presence of the RGD motif does not seem to be the case for HPeVs, where the RGD-lacking HPeV3 is significantly more associated with severe symptoms compared to the other genotypes. Possibly HPeV3 uses a different RGD-independent pathway than shown for CAV9 and E9. Previous studies show that the RGD-lacking CAV9 was not able to infect the A549 cell line, which can be efficiently infected by HPeV3 (16, Chapter 2).

Primary cell culture systems in which cells are used representing the tissue exposed to the viral infection provide a good alternative for pathogenicity studies. In this thesis we introduced the human airway epithelial as a primary cell culture system for HPeVs, as transmission of HPeVs is supposed to be oral-fecal, where the respiratory epithelium is the initial tissue exposed to HPeVs. We showed efficient replication of HPeV3 and we were the first to propagate HPeV9 and HPeV14, all three lacking the RGD motif. The replication of HPeVs in HAE suggests an important role of the respiratory epithelium in HPeV infections. For EV71 pathogenesis studies in rhesus monkeys different routes of infection were studied. Monkeys infected via the respiratory route showed more severe lesions in the CNS compared to monkeys infected via the digestive route (17). This route of infection in severe infections could also be the case for HPeV3, supported by the efficient replication in the HAE cells. In addition, the yet unidentified RGD-independent receptor should be expressed on the respiratory epithelium. Therefore, HAE provides a new tool for characterization of receptors for the RGD-lacking HPeVs. We found one donor in which replication of HPeV1 and HPeV3 was not detected (Chapter 4), indicating the importance of donor screening and selection for further pathogenesis and receptor studies. This lack of replication could be because of the missing (co-) receptor for viral binding or entry, and thus comparison of the different donors using micro-array could lead to identification of the unknown HPeV receptor in healthy human tissue.

The mechanism of entry of HPeVs remains unclear. Integrins are an important receptor for HPeV1 cell attachment as well for E1, CAV9 and E9. For these HEVs the integrins serve as a primary receptor, as co-receptors are needed for capsid alterations and thus infectious entry (16, 18–23). The usage of co-receptors and the internalization of virus is a complex phenomenon wherein a virus may use different mechanisms to enter different cell types. Possibly HPeV1 uses, next to the integrins as their primary receptor, one or more co-receptor(s) for entry into the cells. HPeV1 infection can be fully inhibited by blocking of the RGD motif, indicating that the RGD-integrin binding is essential for binding, but not necessarily also for entry. The first and only study on HPeV1 entry suggested the role of other molecules involved in entry, due to the lack of co-localization of HPeV1 and the integrin subunits during the entry process (11). In case of CAV9 the primary interaction

with the  $\alpha\beta 3$  integrin is followed by utilizing the major histocompatibility complex class I (MHC-I), which is involved in the internalization of the virus. One subunit of the MHC-I complex is the  $\beta 2$ -microglobulin ( $\beta 2M$ ), used by CAV9 (12), as well as by E11 (24) and EV7 (25). Many HEVs use decay accelerating factor (DAF or CD55) as a cell surface receptor; EV7, EV70, CBV3, CBV5, and CAV21 (26–29), where CVB3 requires CAR as co-receptor, and CAV21 requires the intracellular adhesion molecule ICAM-1 for cell entry (28), both members of the Ig superfamily. In case of human rhinoviruses A and B, ICAM-1 seems to be functioning as a single receptor involved in binding and entry (reviewed in (30)). As several HEVs use similar (entry) receptors, this knowledge provides a tool for the identification of (co-) receptors for HPeVs as they might use common receptors, by blocking the known receptors with Abs or peptides.

### **Lack of direct HPeV3 Ab neutralization**

For most HEVs it has been shown that antibodies play a major role in controlling viral spread and thus disease severity. Patients with a deficient humoral immune response, such as X-linked agammaglobulinemia, are at great risk of chronic enteroviral infections (31) where prolonged viral replication was shown (32, 33). In neonates it has been shown that the lack of maternal antibodies is a risk for severe disease (34). In our studies we could not detect HPeV3 protective antibody titers in two adult donors during and one year after infection. In addition, IVIG manufactured between 2005-2010 contained high neutralizing Ab titres against HPeV1, but could hardly neutralize HPeV3 strains. In contrast to our data on low HPeV3 seroprevalence in Finland and the Netherlands, Japan is the only country that reported high seroprevalence for HPeV3; this could be due to different methods of scoring Ab neutralization. In our studies we did see some inhibition of CPE in our serum neutralization assays (chapter 3), but full neutralization as seen for the other genotypes was absent. Scoring for full neutralization will lead to lower seroprevalence numbers, compared to scoring on inhibition. Although if Ab neutralization had been scored by 50% inhibition, this would still have led to much lower (non-protective) titers for HPeV3 Abs compared to Ab titers found against the other genotypes in our studies. To further study neutralization of HPeV3 *in vitro* we isolated three different HPeV3 mAbs. These Abs were specific in HPeV3 viral binding, but we could still not detect efficient neutralization of viral infection. Taken together, all this seems to indicate that Ab protection against HPeV3 might fail, even in the presence of specific aHPeV3 Abs. This possibly indicates that the Abs are not able to efficiently reach the neutralizing epitope and thus cannot interfere with receptor binding. A highly speculative theory to explain this is the membrane hijacking recently shown for the picornavirus Hepatitis A (HAV) (35). HAV released from hepatocytes is cloaked in host cell derived membranes, thereby protecting the virions against antibody-mediated neutralization, while they are fully infectious. These enveloped HAV particles were only

found in plasma and not in faeces indicating that this membrane hijacking is cell type dependent. HAV replication is well established in the liver, indicating that the production of these enveloped viruses only occurs in the liver, from where the virus can spread via the blood protected from antibody neutralization. During viral replication in the intestine these enveloped viruses seem not to be produced as the virus isolation from faeces only reveals non-enveloped viruses. To further investigate this phenomenon the viral supernatants from different (primary) cell lines including our HAE, need to be tested for virus presence in fractions of a gradient loaded with the supernatants, as the enveloped virus particles will appear in a different fraction based on size segregation. During our Cryo-EM studies we never detected any of these envelopes, which is possibly due to the purification protocol obtaining large amounts of high concentrated HPeV3, maybe losing or disrupting the membrane-containing viral particles. A second hypothesis to explain neutralization failure is that the Abs are not able to neutralize viral infection in *in vitro* experiments. For FMDV it has been shown that mAbs against conformational epitopes passively protect neonatal syngeneic (BALB/c) mice at dilutions that could not neutralize virus infection *in vitro*. This indicated that opsonisation and subsequent phagocytosis of the Ab-virus complex play a major role in the immune response against FMDV (36). Possibly this antiviral effect of Abs is as well the case for the immune response against HPeV3, where virus-Ab complexes activate complement proteins that bind to the virus, leading to opsonisation by phagocytes containing receptors for complement. Another possible mechanism could be antibody dependent cell-mediated cytotoxicity (ADCC). The antibody binds membrane surface antigens of the infected cells, inducing cell lysis mediated by natural killer cells or macrophages. Whether these Abs are able to protect against HPeV3 infection *in vivo*, animal models are needed for further research. It has been shown that the availability for animal models for HPeV infection seems to be limited. Newborn mice inoculated with HPeV1 and HPeV2 were only infrequently infected, and cynomolgus monkeys showed no neuropathological changes after 30 days of observation (37). However, HPeVs were detected in feces of monkeys with diarrhea (38), which suggests that they could serve as an infectious model for studying Ab protection of HPeV3.

The isolation of the highly neutralizing Abs against HPeV1 (AM18, AM28) does show the importance of direct neutralization of infection by the immune system for HPeVs. Both mAbs showed high cross-neutralization with HPeV2. This cross-neutralization indicates that HPeV1 and 2 share similar neutralizing epitopes, as discussed in chapter 7. The AM18 Ab was cross neutralizing HPeV2, 4, 5 and 6 infections, but not with HPeV3. We even detected cross reactivity between CAV9 and HPeV1 with the AM18 mAb, a feature unexpected in a single monoclonal antibody against a human picornavirus. This observed cross-reactivity of two monoclonal Abs gives a good perspective for the development of



cross-protective anti-HPeV treatment (as discussed below). This cross-reactivity of Abs against HPeV1 with HPeV2 could nicely explain why high seroprevalence against HPeV2 is found in the Netherlands and Finland (86-99%) (Westerhuis *et al.*, 2013), where HPeV2 infections are hardly detected (39–41). Mapping the neutralizing epitopes of these Abs revealed that the AM18 was inhibiting viral infection directly by binding the integrin RGD motif, again showing the importance of the RGD motif in HPeV1 viral infection (Chapter 7). The AM28 did not show inhibition of viral infection by direct interaction with the RGD. Reconstruction suggests that the AM28 Ab staples the neighbouring pentamers together, thereby stabilizing the whole capsid. We showed that the HPeV1 labelled with AM28 raises the temperature for capsid destabilization, preventing uncoating of RNA from the capsid on cell entry. We identified several contributions from both VP0 and VP3 involved in the Fab binding by homology modelling. To further look into contribution of the specific residues in the different loops *in vitro* responsible for the neutralization, alanine scanning methods needs to be performed.

### HPeV infection; the need for treatment

In the previous years the clinical importance of HPeVs has been revealed. Currently fast diagnostics are available and the implementation of HPeV viral diagnostics is increasing among laboratories. Despite the fact that HPeV infections can be severe and life threatening, no antiviral treatment is available. The groups most at risk for severe HEV and HPeV infections are neonates as their immune response is not fully developed, and patients with a humoral immune deficiency (31, 34, 42, 43). In neonates an option for supportive treatment is the use of intravenous immunoglobulins (IVIg), while in patients with a humoral immune deficiency IVIg is already used as a replacement therapy. A pitfall of this treatment is that IVIg remains unspecific (31) and Igs against newly introduced or rare pathogens are not covered. We showed that the IVIg batches contained only a very low concentration of neutralizing Abs against HPeV3 *in vitro*, possibly resulting in failure of treatment. An option for antiviral treatment of HEV and HPeVs would be the use of specific monoclonal Abs. HPeV is a good candidate for development of mAbs due to the restricted group of genotypes. In this thesis we describe two good candidates for Ab treatment of HPeV1 infections (Chapter 6 and 7). One candidate even gives cross-neutralization to the other RGD containing genotypes. Unfortunately, the three mAbs specifically binding HPeV3 showed no *in vitro* neutralization of infectivity. Although we found some viral inhibition of HPeV3 infection, revealing that possibly nAbs against HPeV3 do exist, leaving a great challenge finding these Abs.

Although the different antigenic sites and the observed cross reactivity makes HPeVs a good target for the development of therapeutic human Abs, the lack of (cross-) neutralization of HPeV3 might be a pitfall. Next to Ab therapy other options would be capsid inhibitors

or replication inhibitors. A promising drug in HEV treatment was the capsid inhibitor pleconaril, showing clinical activity in some patient groups (reviewed in (44–46)). However, even when the compound for some reason would return to the market, it will not be beneficial in the treatment of HPeVs since our group showed that HPeV1 and HPeV3 were resistant against pleconaril *in vitro* (47). Compounds with antiviral activity against HEV will not automatically possess antiviral activity against HPeVs. Although the genome structure looks similar to that from HEVs, the genome variety of HPeVs is extensively different. During the maturation cleavage of VP0 into VP4 and VP2 does not occur in HPeVs. Secondly, the viral capsid of HPeVs are shown to be much more smoother compared to HEVs ((12), Chapter 7, 9), containing different features of the capsid compared to HEVs. Therefore, capsid inhibitors against HEV will probably not inhibit HPeVs. Next to capsid inhibitors a wide range of processes in the viral life cycle are potential targets in the development of antiviral treatment. However in the non-structural proteins again differences between HEVs and HPeVs are shown. In the case of HPeVs 2A lacks the proteolytic activity seen in other picornaviruses; furthermore it seems that only one protease, 3Cpro, is involved in processing (48–50). Phylogenetic analyses of the 3Dpol region showed only a low degree of identity of HPeVs compared to other picornaviruses (48). For HEVs several compounds are describes showing broad picornaviral activity (46), although these compounds never have been tested for antiviral activity against HPeVs. Given the differences between HEVs and HPeVs it remains to be seen whether these compounds show antiviral activity against HPeVs.

## In conclusion

HPeVs are among the most frequently detected picornaviruses in the world, and are nowadays accepted as an important viral agent causing severe CNS symptoms in neonates. These severe infections are most pronounced for HPeV3. In this thesis we showed that the lack of direct neutralization might play a role in these severe infection. Thereby we showed that the respiratory epithelium might play an important role in HPeV3 infection and entry. The first step towards more answers is the identification of the receptor binding epitopes and the receptor for HPeV3. We introduced a primary respiratory cell culture tool for HPeV infection expressing the RGD independent receptor for HPeVs, as culturing of the RGD-lacking HPeV types was successful. Together with blocking infection with viral HPeV3 peptides, available microarray techniques, and cryo-EM techniques, receptor identification should be feasible.

The development of cross-neutralizing HPeV mAbs displaying high neutralizing titers against several HPeVs shows promising results for monoclonal Ab based therapy, although this approach might not yet work against HPeV3 for which no highly neutralizing Abs

could be identified. This underlines the need for elucidating epitopes for binding and neutralization of HPeV3, as well as for further investment in developing anti-picornaviral therapy with broad antiviral activity, including activity against HPeVs.

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A.

## Appendices



## Samenvatting voor niet ingewijden

Virussen zijn ziekteverwekkers opgebouwd uit genetisch materiaal, het genoom, dit kan RNA of DNA zijn, omhuld door een eiwitmantel, het capsid genoemd. Virussen zijn niet in staat zelfstandig te vermenigvuldigen, hiervoor is een gastheercel nodig. Na binding van een receptor (eiwit) op de gastheercel kan het virus de cel in. Eenmaal in de cel worden de cellulaire processen gebruikt om het genetisch materiaal te vermenigvuldigen en eiwitten te produceren, waaruit nieuwe virusdeeltjes opgebouwd kunnen worden. Deze nieuw geproduceerde virusdeeltjes verlaten de cel om weer nieuwe cellen te infecteren.

Een grote groep virussen zijn de picornavirussen, dit zijn de kleinste en oudste RNA virussen bekend. Binnen deze groep vallen de humane enterovirussen (HEV) en de humane parechovirussen (HPeV). Het genoom van deze virussen is opgebouwd uit 7300 nucleotiden (de genetische code), die coderen voor 10 eiwitten. Drie eiwitten (VP0, VP3, VP1) waarvan elk 60 kopieën vormen het capsid. De zeven andere eiwitten zijn nodig voor het vermenigvuldigingsproces. De groep van HPeVs bestaan uit 16 verschillende genotypen, die worden ingedeeld op basis van verschillen in de genetische code. De eiwitten zijn weer opgebouwd uit bouwstenen, peptides genaamd. Een belangrijk motief in deze bouwstenen is het RGD motief. De genotypes 1, 2, 4, 5 en 6 hebben dit motief, dat belangrijk is voor het binden van het virus aan de receptor op de cel. De genotypen HPeV3, 7-16 bevatten dit motief niet, en kunnen dus niet aan de cel binden via dit motief. Het motief dat deze virussen gebruiken om aan de cel te binden, en via welk cellulair eiwit dit gaat, is onbekend. Humane parechovirussen worden meestal geassocieerd met milde symptomen, waaronder luchtweginfecties en infecties van het maag-darmkanaal, maar ook met ernstiger ziektebeelden zoals verlammingen, infectie van het centraal zenuwstelsel, en ernstig algeheel ziek zijn bij pasgeborenen. In reactie op virussen wordt in het lichaam het immuunsysteem actief. Een deel van die afweer is de productie van antistoffen door B-cellen. Deze antistoffen zijn specifiek en kunnen op verschillende manieren bijdragen aan het tegengaan van de virale infectie. Ze binden aan het virus waardoor het immuunsysteem in staat is het virus verder kapot te maken, of ze binden aan het virus waardoor de virus-cel (receptor) binding tegen gegaan wordt waardoor het virus zich niet verder kan verspreiden. Dit laatste wordt virusneutralisatie genoemd. Na een infectie komt deze antistofproductie op gang, antistoffen specifiek gericht tegen dit virus kunnen worden aangetoond in het bloed en wordt je 'seropositief' tegen het specifieke virus. In pasgeboren baby's is de immunrespons nog niet volledig ontwikkeld, wat ze vatbaar maakt voor infecties. Tijdens de zwangerschap krijgen baby's via de placenta antistoffen van de moeder mee, en deze antistoffen bieden bescherming tegen infecties in de eerste maanden na geboorte.

In de pathogenese van virussen, daarmee wordt bedoeld het infectieproces en de schade die het virus hiermee aanricht, spelen verschillende factoren een rol. Allereerst welke cel of orgaan het virus kan infecteren; dit is afhankelijk is van het type cel en de eiwitten (receptoren) aanwezig op deze cel. De voorkeur van virussen voor een cel wordt het cel tropisme van dat virus genoemd. Ten tweede is de bescherming van het immuunsysteem tegen de virus infectie belangrijk. In dit proefschrift wordt het cel tropisme en de antistofbescherming tegen HPeVs onderzocht.

Receptor binding speelt een grote rol in de pathogenese van het virus. Allereerst wilden we weten welke laboratorium cellijnen geïnfecteerd konden worden met de verschillende virus types. Hierbij hebben we naast de standaard meetgraad voor virusinfectie van de cel, het zogenaamde 'CPE' (cytopathic effect, het effect van een virusinfectie op de vorm van de cellaag), een tweede meetmethode geïntroduceerd (Hoofdstuk 2). Deze meetmethode bestaat uit het meten van de productie van virus door de detectie van het aanwezig genetisch materiaal van het virus. Door het introduceren van deze nieuwe meetmethode zagen we dat er meer soorten cellen geïnfecteerd werden dan we voorheen hadden gezien. We zagen ook dat er al virusvermeerdering plaatsvindt zonder dat het CPE zichtbaar is.

Om de pathogenese van HPeV1 en HPeV3 te vergelijken is er een selectie gemaakt van verschillende HPeV1 en HPeV3 isolaten van patiënten met milde en ernstige symptomen gedurende een HPeV1 of 3 infecties (Hoofdstuk 3). Deze isolaten zijn gebruikt om cellen die ooit geïsoleerd zijn uit menselijk darmtumor en longtumor weefsel, en een cellijn afkomstig van cellen uit het centraal zenuwstelsel te infecteren, om te kijken naar het verschil in cel tropisme om zo een link te kunnen leggen met ernst van ziekte. We zagen dat de HPeV3 stammen met name geïsoleerd uit patiënten met ernstige symptomen beter replicateerden op de neurale cellijnen in vergelijking tot de HPeV1 stammen, maar verder kon er geen duidelijke relatie gelegd worden tussen cel tropisme van het virus en de ernst van ziekte in de patient. De cellijnen gebruikt zijn allemaal continu groeiende cellijnen geïsoleerd uit tumoren, en dezen zijn niet representatief voor het gezonde weefsel in de mens. In hoofdstuk 4 beschrijven we een humaan luchtweg epitheel cel systeem wat precies lijkt op het opperste luchtwegweefsel in de mens. Dit systeem kan heel goed gebruikt worden om een virusinfectie in de luchtwegen van de mens te bestuderen. In dit humaan luchtweg epitheel is ook de replicatie van HPeVs gemeten. In tegenstelling tot wat er waargenomen is in standaard cellijnen, zagen we dat HPeV3 beter replicateerde dan HPeV1. Daarbij zagen we replicatie van HPeV9 en HPeV14 in humaan luchtwegepitheel, terwijl deze stammen niet konden repliceren in standaard cellijnen. Net als HPeV3, hebben HPeV9 en HPeV14 genetisch niet het RGD motief, maar kunnen ze wel het luchtwegepitheel infecteren. Dit betekent dat in dit luchtwegepitheel de nog onbekende HPeV receptor aanwezig is. Het verschil in groei van de HPeV typen tussen de verschillende cellijnen laat zien hoe belangrijk

de keuze is van cellijnen in verder (receptor) onderzoek.

Studies hebben aangetoond dat er veel HPeV1 antistoffen aanwezig zijn in het bloed van mensen van verschillende leeftijdsgroepen in diverse landen, maar over de andere circulerende HPeV types zijn geen data bekend. In hoofdstuk 5 wordt er gekeken naar de aanwezigheid van antistoffen tegen HPeV1 t/m 6 in een selectie van verschillende leeftijdsgroepen uit Nederland en Finland. Het bleek dat in de meeste volwassenen beschermende neutraliserende antistoffen tegen HPeV1 en HPEV2 konden worden aangetoond (in 86%-99%). De hoge positiviteit tegen HPeV2 was niet verwacht, omdat in beide landen geen HPeV2 infecties worden gezien. De seroprevalentie, dus het aantoonbaar zijn van specifieke antistoffen in het bloed, tegen HPeV4 was hetzelfde in beide populaties (60% in Finland en 62% in Nederland), terwijl de seroprevalentie tegen HPeV5 (75% vs. 35%) en HPeV6 (74% vs. 57%) hoger was in Nederland vergeleken met Finland. In beide landen vonden we lage seroprevalenties tegen HPeV3, 4% in Finland en 8% in Nederland. Dit was wederom niet verwacht omdat HPeV3 infecties in Finland en Nederland zeer regelmatig worden gevonden, waarbij er dus verwacht wordt dat er antistoffen aantoonbaar zullen zijn in de populatie. Verder bleek dat specifieke HPeV3 antistoffen vervaardigd in dieren of geïsoleerd uit mensen het virus niet konden neutraliseren. Deze data laten zien dat er of weinig neutraliserende antistoffen aanwezig zijn in de verschillende populaties of dat de antistoffen het virus niet goed kunnen binden, waardoor er nog steeds binding van het virus plaats kan vinden aan de receptor op de cel. Bij de andere HPeV types worden de beschermende specifieke antistoffen wel gevonden in het bloed, en kunnen ze het desbetreffende virus type neutraliseren, waardoor er bescherming optreedt tegen latere infectie met dit type. De afwezigheid van beschermende antistoffen tegen HPeV3 in de bevolking kan dus ook verklaren waarom pasgeborenen geïnfecteerd raken met HPeV3: er zijn geen beschermende antistoffen van de moeder aanwezig tegen HPeV3.

In het tweede deel van deze thesis worden de hiervoor beschreven bevindingen met betrekking tot neutralisatie verder uitgelicht. Door middel van het enten van konijnen met HPeV1 en 3 zijn er tegen HPeV1 en HPeV3 antistoffen gemaakt, zogenaamde polyclonale antistoffen, dit is een mix van antistoffen gericht tegen verschillende oppervlakte delen van het virus. Daarnaast hebben we met een geavanceerde methode ook menselijke antistoffen gemaakt door een B cel, die gericht zijn tegen een deel van het oppervlakte van het virus, zogenaamde monoklonale antistoffen. Dit resulteerde in twee antistoffen gericht tegen HPeV1 en drie antistoffen gericht tegen HPeV3 (Hoofdstuk 6 & Hoofdstuk 8). De verschillende antistoffen zijn allemaal getest voor neutralisatie en binding van de verschillende typen HPeV1-6. De polyclonale antistof gericht tegen HPeV1 laat kruisbinding zien aan de verschillende types, maar was ook in staat om de verschillende typen te neutraliseren, dit is te verklaren

aan de mix van antistoffen gericht tegen verschillende oppervlakte delen van het virus. In tegenstelling tot wat algemeen gedacht wordt, dat de monoclonale antistoffen gericht zijn tegen één deel van het virus en dus specifiek zijn voor binding en neutralisatie van 1 specifiek genotype, waren beide monoclonale antistoffen in staat om zowel HPeV1 als HPeV2 te neutraliseren, waar een van de twee antistoffen (Antistof 1) zelfs in staat was om ook de genotypen HPeV4, 5 en 6 te neutraliseren. Beide HPeV1 antistoffen waren niet in staat om het type HPeV3 te neutraliseren. Via een specifieke methode (peptide scanning) blijkt dat de Antistof 1 bindt aan het eerder genoemde RGD eiwit en dus de binding van het virus aan het receptor eiwit blokkeert. Voor de identificatie van het virus deel waaraan Antistof 2 bindt was een meer geavanceerde techniek nodig, waarbij de binding van de antistof aan het virus in beeld wordt gebracht door middel van cryo-elektronen microscopie. Hieruit blijkt dat de antistof twee verschillende plekken op twee verschillende virus eiwitten bindt, VP0 en VP3, en dus de samenstelling van het hele virus nodig heeft om te kunnen binden (Hoofdstuk 7).

De polyclonale HPeV3 antistof was in staat om alle typen HPeV1-6 te binden maar was niet goed in staat de virus infectie te neutraliseren, dit was alleen mogelijk met een heel hoge concentratie antistof. De monoclonale antistoffen lieten alleen specifieke binding zien aan HPeV3, en geen neutralisatie. Mogelijk kan de antistof de virusinfectie (in een celkweek systeem) niet neutraliseren. Het in beeld brengen van de structuur van HPeV1 en HPeV3 kan meer inzichten geven in de verschillen in receptor en antistofbinding tussen deze typen. In deze thesis hebben we met behulp van cryo-elektronen microscopie een structuur weergave van HPeV3 gegenereerd (Hoofdstuk 9). Verder onderzoek is nodig om meer gedetailleerde uitspraken te doen over de verschillen tussen HPeV1 en HPeV3.

In deze thesis hebben wij aangetoond dat HPeV3 verschilt in zowel cel tropisme als antistof neutralisatie ten opzichte van de andere genotypen. Dit is waardevolle informatie in verder onderzoek naar de pathogenese van HPeV3 en de ontwikkeling van de momenteel nog niet beschikbare behandeling en medicijnen tegen HPeV infecties.

## Curicullim Vitae

Brenda Westerhuis was born on 19th of februari 1986 in Naarden, the Netherlands. In 2004 she graduated the 'Atheneum' Nature and Health from the Baken Park Lyceum in Almere. After graduation she went to study Biomedical sciences at the University of Amsterdam. In 2008 she finished her Bachelor with a traineeship at the Laboratory for Experimental Oncology and Radiobiology, Academic Medical Center in Amsterdam. She did her first Master's traineeship at the department of Medical Microbiology, Laboratory of Clinical Virology, Academic Medical Center Amsterdam, working on the clinical and molecular insights of human parechoviruses. In 2010 she finished her Masters with an internship at the Dutch Vaccin Institute in Bilthoven on host response to lipopolysaccharide mutants of *Neisseria meningitidis*. After receiving her Masters degree in Biomedical Sciences she started her PhD project at the department of Medical Microbiology, Laboratory of Clinical Virology working on the characteristics of HPeVs. During her PhD, she made in total a 4 months workvivist to the University of Helsinki, under the supervision of prof. Sarah Butcher. Working on cryo-EM structural studies of HPeV1 and HPeV3. In 2014 she will continue her work on picornaviruses as post-doc at TIB Molbiol in Berlin, Germany.

## **List of publications**

**Clinical characteristics of human parechoviruses 4-6 infections in young children.** Pajkrt D, Benschop KS, Westerhuis B, Molenkamp R, Spanjerberg L, Wolthers KC., *Pediatr Infect Dis J.* 2009 Nov;28(11)

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**Prevalence and clinical course in invasive infections with meningococcal endotoxin variants.** Rodenburg GD, Fransen F, Bogaert D, Schipper K, Groenwold RH, Hamstra HJ, Westerhuis BM, van de Beek D, van der Ley P, Sanders EA, van der Ende A., *PLoS One.* 2012;7(11)

**Specific cell tropism and neutralization of human parechovirus types 1 and 3: implications for pathogenesis and therapy development.** - Westerhuis BM, Koen G, Wildenbeest JG, Pajkrt D, de Jong MD, Benschop KS, Wolthers KC. *J Gen Virol.* 2012 Nov;93:2363-70

**Prevalence and clinical course in invasive infections with meningococcal endotoxin variants.** Rodenburg GD, Fransen F, Bogaert D, Schipper K, Groenwold RH, Hamstra HJ, Westerhuis BM, van de Beek D, van der Ley P, Sanders EA, van der Ende A. *PLoS One.* 2012; 7(11)

### **Human Parechovirus seroprevalence in Finland and the Netherlands.**

Brenda Westerhuis\*, Pekka Kolehmainen\*, Kimberley Benschop, Noora Nurminen, Gerrit Koen, Marjaleena Koskiniemi, Olli Simell, Mikael Knip, Heikki Hyöty, Katja Wolthers and Sisko Tauriainen. *J Clin Virol.* 2013 Sep;58(1):211-5

**Growth Characteristics of Human Parechovirus 1 to 6 on Different Cell Lines and Cross-neutralization of Human Parechovirus Antibodies; a Comparison of the Cytopathic Effect and Real Time PCR.** - Brenda M. Westerhuis, Sara C.M. Jonker, Sandhia Mattao, Kimberley S. M. Benschop and Katja C. Wolthers. *Virology Journal* 2013, 10:146

## **Submitted for Publication**

**Replication of Human Parechovirus in 3D Human Airway Epithelial Cell models -**  
B.M. Westerhuis, K.S.M. Benschop, R.E. Jeeninga, M.D. de Jong and K.C. Wolthers  
Human memory B cells producing potent cross-neutralizing antibodies against Human

**Human memory B cells producing potent cross-neutralizing antibodies against human parechovirus; implications for prevalence, treatment and diagnosis** B.M. Westerhuis, K.S.M. Benschop, G. Koen, Y. Claassen, A.Q. Bakker, K.C. Wolthers and T. Beaumont

**Structural basis of human parechovirus neutralization by human monoclonal antibodies.** S. Shakeel, B. M. Westerhuis, G. Koen, A.Q. Bakker, Y. Claassen, A. Ora, T. Beaumont S. J. Butcher and K. C. Wolthers

**Genetic and antigenic structural basis for natural resistance of Echovirus 11 to pleconaril in an immuno-compromised patient.** Kimberley Benschop, Joanne Wildenbeest, Gerrit Koen, Rene Minnaar, Formijn van Hemert, Xiomara Thomas, Brenda Westerhuis, Dasja Pajkrt, Peterhans van den Broek, Ann Vossen, Katja Wolthers.

## AMC Graduate School for Medical Sciences PhD Portfolio

Summary of PhD training, teaching and parameters of esteem

Name PhD student: B.M. Westerhuls

PhD period: 2010 - 2014

Name PhD supervisor: K.C. Wolthers

<b>Courses</b>	<b>Year</b>	<b>Workload (ECTS)</b>
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### Graduate School

• The AMC world of Science	2010	0,7
• Pubmed Course	2010	
• Project Management	2012	0,6
• Mass Spectrometry, Proteomics and Protein Research	2011	2,1
• Clinical Epidemiology	2010	0,9
• Scientific Writing in English for Publications	2010	1,5
• Infectious Diseases	2010	1,3

<b>Conferences</b>	<b>Year</b>	<b>Workload (ECTS)</b>
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• Annual meeting of European Society for Clinical Virology	2011	1,0
• Europic 2012	2012	1,5
• Positive Strand Meeting	2013	1,25
• Meeting of European Society for Virology	2013	1,0
• Europic 2014	2014	1,5

<b>Presentations</b>	<b>Year</b>	<b>Workload (ECTS)</b>
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### Oral

• Annual meeting of European Society for Clinical Virology <i>Specific Cell Tropism and Neutralization Capacity of HPeV3: Implications for Pathogenesis</i>	2011	0,5
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**Posters**

- |  |      |     |
|--|------|-----|
| • Europic 2012<br><i>Specific Cell Tropism and Neutralization</i><br><br><i>Capacity of HPeV3: Implications for Pathogenesis</i> | 2012 | 0,5 |
| • ESV 2013<br><i>Polyclonal and monoclonal antibodies against human parechovirus 1 and 3</i>                                     | 2013 | 0,5 |
| • Positive Strand Meeting<br>3D Reconstruction of the Human Parechovirus 3<br><br>Structure                                      | 2013 | 0,5 |
| • Europic 2014<br>Replication of Human Parechovirus in 3D Human<br><br>Airway Epithelial Cell models                             | 2013 | 0,5 |

<b>Seminars</b>	<b>Year</b>	<b>Workload (ECTS)</b>
-----------------	-------------	------------------------

- |                               |           |     |
|-------------------------------|-----------|-----|
| • Weekly department seminars  | 2010-2014 | 5,0 |
| • Monthly department seminars | 2010-2013 | 1,0 |

<b>Lecturing</b>	<b>Year</b>	<b>Workload (ECTS)</b>
------------------	-------------	------------------------

- |   |           |     |
|---|-----------|-----|
| • Supervising<br>Darscha Amarthalingam<br><i>Project Title: Prevalence and seroprevalence of Human Enterovirus and Human Parechovirus in Malawi</i> | 2013/2014 | 2,0 |
|---|-----------|-----|

<b>Funding</b>
----------------

- European Society for Clinical Virology - Travel Grant 2011
- European Society for Clinical Virology - Training Grant
- EMBO Short term fellowship

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