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

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

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Abstract

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

Human parechoviruses (HPeVs) are small, non-enveloped, single-stranded positive-sense RNA viruses within the genus *Parechovirus* in the family *Picornaviridae*. HPeV1 and HPeV2 were originally described as echoviruses 22 and 23 within the genus *Enterovirus*, based on their similar cytopathic effect (CPE) on monkey kidney cells. 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. 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. HPeV1, HPeV2 and HPeV4-6 are all mainly associated with milder symptoms; HPeV3 infections cause central nervous system (CNS) infections and neonatal sepsis.

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. 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. The differences in *in vitro* growth characteristics 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. 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. 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.

In addition to differences in clinical symptoms, children infected with HPeV3 are significantly younger (<2 months) than children infected with HPeV1 (>6 months). This might suggest a lack of protection by maternal neutralizing antibodies (nAb) in newborns against HPeV3. For HPeV3, the seroprevalence among women of child bearing age in Japan is 68%, which is lower than the seroprevalence in Finland for HPeV1 in adults (99%). For EVs, it has been shown that lack of maternal antibodies is a risk factor for severe disease in infants. 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.
In this study, we investigate *in vitro* replication kinetics of HPeV1 and HPeV3 virus strains by measuring viral RNA in the supernatant of a range of cell lines by real-time PCR, and relate the kinetics to disease severity. In addition, neutralization capacity of available type-specific antibodies and IVIG was tested *in vitro* to study protection against HPeV1 and HPeV3 infection.

## Results

### Replication kinetics of HPeV1 and HPeV3 patient strains

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

<table>
<thead>
<tr>
<th>Patient*</th>
<th>Age (months)</th>
<th>GI</th>
<th>Resp</th>
<th>SLI</th>
<th>CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1-1</td>
<td>4.7</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P1-2</td>
<td>9.9</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P1-3</td>
<td>6.0</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P1-4</td>
<td>5.6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P1-5</td>
<td>4.8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>P1-6</td>
<td>0.7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>P3-1</td>
<td>0.9</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P3-2</td>
<td>10.6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P3-3</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<tr>
<td>P3-4</td>
<td>10.8</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>P3-5</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>P3-6</td>
<td>0.2</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>P3-7</td>
<td>1.7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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

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

Six HPeV1 strains and seven HPeV3 strains isolated from 13 stool samples were selected according to the clinical manifestations and severity of disease documented for the patients (Table 1) and were cultured with the same input per cell culture (m.o.i. 0.001). Of the HPeV1-
infected children, patients P1-1 to P1-4 showed mild gastrointestinal and/or respiratory symptoms; P1-5 in addition had sepsis-like illness (SLI) and P1-6 suffered from SLI with CNS involvement.\textsuperscript{8,20} In the HPeV3 group, P3-1 and P3-2 had mild disease without SLI or CNS symptoms, P3-3 had SLI without CNS involvement, P3-4 had CNS infection without SLI, and P3-5, P3-6 and P3-7 had SLI and CNS involvement.

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

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

Neutralization capacity

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

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

<table>
<thead>
<tr>
<th>HPeV strain</th>
<th>aHPeV1*</th>
<th>Antibody aHPeV3†</th>
<th>None</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPeV1 Harris‡</td>
<td>-</td>
<td>4+</td>
<td>4+</td>
</tr>
<tr>
<td>HPeV2-751312‡</td>
<td>4+</td>
<td>4+</td>
<td>4+</td>
</tr>
<tr>
<td>HPeV3-150237‡</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>HPeV4-251176‡</td>
<td>4+</td>
<td>4+</td>
<td>4+</td>
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<td>HPeV5-552322‡</td>
<td>4+</td>
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</tr>
<tr>
<td>HPeV6-550389§</td>
<td>4+</td>
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</tr>
</tbody>
</table>

*Antibody dilution for HPeV1 was 1 : 10.
†Antibody dilution for HPeV3 was 1 : 2.
‡100 TCID<sub>50</sub> infection on HT29 (HPeV1, HPeV2, HPeV4, HPeV5) and Vero (HPeV3) cells.
§1000 TCID<sub>50</sub> infection of HT29.
Figure 2. Neutralization ability of aHPeV3 antibody. IFA for detection of HPeV3-150237, A308-99 and P3-2 after HPeV3 A308-99 antibody neutralization. A standard neutralization assay was performed: the HPeV3 strains were pre-incubated with the polyclonal A308-99 antibody and used to infect Vero cells. Cells were stained at day 7 with polyclonal antibody against HPeV3, and secondary FITC-labelled goat IgG antibody (green). The nuclei of the cells are stained with DAPI (blue).

Figure 3. Immunofluorescence showing HPeV1 and HPeV3 antibody binding. IFA for detection of HPeV1 infection in HT29 cells and HPeV3 infection in Vero cells. When a CPE of 2+ (25-50% of cells infected) was reached, cells were stained with polyclonal antibodies against HPeV1 and HPeV3, and secondary FITC-labelled goat IgG antibody (green). The nuclei of the cells are stained with DAPI (blue).
To test the neutralization capacity of IVIG against HPeVs, six different IVIG batches from 2005, 2008, 2009 and 2010 were used for neutralization. High neutralizing titres against HPeV1 were found (>1:1280), while neutralization titres against HPeV3-150237 and HPeV3 A308-99 were very low (<1:20 and <1:40, respectively) (Table 3). In contrast, IVIG contained high nAb titres against the other HPeV genotypes 2, 4, 5 and 6 (>1:320) (data not shown). No substantial differences were shown between the IVIG batches from the different years.

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

<table>
<thead>
<tr>
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<th></th>
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</thead>
<tbody>
<tr>
<td>HPeV1 Harris</td>
<td>1280</td>
<td>1280</td>
<td>2560</td>
<td>1280</td>
<td>1280</td>
<td>1280</td>
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<tr>
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<td>20</td>
<td>20</td>
<td>10</td>
<td>20</td>
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<tr>
<td>HPeV3 A308-99</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>40</td>
<td>40</td>
<td>20</td>
</tr>
</tbody>
</table>

To further investigate neutralization of HPeV3, sera from two different donors, in whom an HPeV3 infection had been detected by PCR in a stool sample from the past year, were tested for neutralization by CPE as well as PCR read-out (Fig. 4). High nAb titres against HPeV1 were found in both donors. Sera of donors 1 and 2 showed nAb titres of 1:256 and 1:4096, respectively, at time point 1, indicating previous infection with HPeV1. After 1 year, the sera of donors 1 and 2 had nAb titres of 1:512 and >1:4096, respectively, indicating that nAbs can still be strong at least 1 year after infection. An 8 log_{10} difference was measured in viral copies by PCR between non-neutralized and neutralized virus infection (Fig. 4a), and virus growth could only be visualized by IFA starting at the indicated serum antibody dilutions (Fig. 4b). However, for HPeV3 these high nAb titres could not be found after infection. At time point 2, 1 year after infection, incomplete inhibition of virus growth could be shown by an antibody titre of 8, while no growth inhibition could be found by antibody titres of 32 and upwards (Fig. 4b). Virus replication measured by PCR decreased with a maximum of 2 log_{10} only at the very low nAb titres (<1:16) (Fig. 4a). Therefore, in two adult donors 1 year after HPeV3 infection, partial neutralization can be found only at very low serum antibody titres (<1:16), which is in contrast to the high serum nAb titres against HPeV1 found in these two adult donors without apparent recent HPeV1 infection.
Figure 4. Neutralization of donor serum samples. An end-point neutralization assay of four different serum samples from two different donors who experienced an HPeV3 infection. Time point 1 serum is taken at the time of infection and time point 2 serum 1 year after infection. (a) The viral copies per PCR present at day 7. (b) Cells were stained at day 7 with polyclonal antibody against HPeV3, and secondary FITC-labelled goat IgG antibody (green). The nuclei of the cells are stained with DAPI (blue). CC, Cell control; VC, virus control.

Discussion

Differences in disease manifestations and severity between EV types can be related to differences in cell tropism and antibody neutralization capability. 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. 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. For HPeV1 it has been shown that, next to VP1, VP0 also contains important antigenic sites. 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 %). 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. 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 and has been present since 1994. 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. Based on the low nAb titres found against HPeV3, despite efficient antibody binding, we now hypothesize that antibody protection against HPeV3 might fail. Therefore, other host and/or viral factors must be involved in susceptibility too, or in protection against infection, and these factors will determine the clinical course of HPeV3 infection in young infants. It could be that the virus structure does not permit antibodies to reach the neutralizing epitope. New mono- and polyclonal antibodies are needed to elucidate the mechanisms of neutralization of HPeV3 and get more insight into potential antiviral drug targets.

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

Methods

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

Virus strains and cultivation
The following HPeV strains were used as laboratory prototypes: HPeV1A Harris, HPeV2-751312, HPeV3-150237, HPeV4-251176, HPeV5-552322 and HPeV6-550389.\(^8{,}^{13,}^{33}\) In addition, a selection of HPeV1 and HPeV3 strains isolated from patients with well-documented clinical syndromes (Table 1) was made. The Japanese patient strain A308-99 was a kind gift from Dr Shimizu, National Institute of Infectious Diseases, Tokyo, Japan.\(^5\) The viruses were passaged two or three times to obtain sufficient virus stocks. HPeV1, HPeV2, HPeV4 and HPeV5 were cultured in the HT29 cell line, HPeV3 in the Vero cell line and HPeV6 in the RD99 cell line and the virus working stocks were stored in aliquots at -80 °C. The virus concentration was determined by the TCID\(_{50}\) and calculated by the method of Reed & Muench.\(^{34}\)
Antibodies
The anti-HPeV (aHHeV) 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 aHHeV3 (A308-99) antibody was a kind gift from Dr Shimizu, National Institute of Infectious Diseases, Tokyo, Japan, prepared as pooled guinea pig serum.

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% CO₂. At day 0, the cell lines were infected with HPeV isolates at an m.o.i. of 0.001 in a volume of 200 µl culture medium for 2 h, after which the non-absorbed virus was removed and replaced with 1 ml maintenance medium (2% EMEM, 2% DMEM) and incubated for 10 days. The low m.o.i. is chosen to elucidate the entire process of the infection cycle of HPeV in cell culture. At days 0, 1, 3, 7 and 10, culture supernatant was removed for RNA extraction and quantitative RT-PCR detection. The supernatant (20 µl) was extracted by automatic extraction using a total nucleic acid isolation kit with the MagnaPure LC instrument (Roche Diagnostics). The RNA was eluted in 50 µl elution buffer and reverse-transcribed as described previously. cDNA (5 µl) was used for real-time PCR using a LightCycler 480 (Roche Diagnostics). The virus copies per PCR were calculated using a standard curve as described previously. 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. All experiments were performed twice with reproducible results.

IFA
Black, clear-bottomed 96-well plates (Greiner) seeded with Vero or HT29 cells were inoculated with a virus solution in 8% EMEM. When a CPE of 2+ (25–50% of cells infected) was observed, the infected cells were fixed with 4% paraformaldehyde (PFA)/PBS for 15 min. The PFA was removed and the cells were washed consecutively three times with PBS, 25 mM NH₄Cl/PBS and PBS. The cells were placed in 1xPBS/0.1% Triton X-100 for 10 min and washed three times with PBS. To avoid unspecific binding, wells were blocked with 1% BSA/PBS for 30 min. The blocking buffer was removed and the cells were incubated with the primary antibodies, aHHeV1 (rabbit, 1:10000; Jackson ImmunoResearch) or aHHeV3 (guinea pig, 1:100; Jackson ImmunoResearch), for 1 h at 37 °C and then overnight at 4 °C. Plates were washed consecutively three times with PBS, 0.1% Tween/PBS and PBS. Either anti-rabbit or anti- guinea pig secondary goat antibody FITC-labelled IgG (15 µg ml⁻¹; Jackson ImmunoResearch) and 0.5 µg DAPI ml⁻¹ (Sigma) were added and incubated for 1 h at 37 °C. Plates were washed three times with PBS. The fluorescence was conserved in 50% glycerol/PBS and examined with a fluorescence microscope (Leica).
Neutralization assay
Antibodies were mixed with the different HPeV1 and HPeV3 virus suspensions containing 100 TCID50 per 50 µl. The following antibody dilutions in 2% EMEM were used: aHPeV1 (1:100); and aHPeV3 (1:10). Secondly, six different IVIG batches [Nanogam, Sanquin, The Netherlands, from 2005, 2008 (two batches), 2009, 2010 (two batches)]31 were used for end-point neutralization of 100 TCID50 per 50 µl HPeV1 and HPeV3. IVIG are batches of pooled IgG extracted from plasma of over 1000 donors. An end-point neutralization was performed with serum from two HPeV3-infected donors at two different time points. The first serum sample was taken during the HPeV3 infection and the second sample 1 year after infection. Mixtures were incubated at 37 °C for 1 h, and were used to inoculate HT29 cells (HPeV1) and Vero cells (HPeV3) on a 96-well plate (200 µl). Virus, cell and antibody controls were included as positive and negative controls. The cells were examined for the appearance of CPE every 24 h for 7 days. At day 7, the medium, containing unbound antibody, was removed and then cells were fixed in the 96-well plate and an IFA was performed. For the patient serum neutralization, the virus copies per PCR were measured by real-time PCR.

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