Clinical and molecular insights into human parechovirus infection

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General introduction
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Human parechoviruses (HPeVs) have been recognized since 1992 as a separate group in the family Picornaviridae on the basis of distinct molecular and biological properties. They have predominantly been associated with mild gastrointestinal and respiratory symptoms. Although more severe symptoms have been associated with HPeV1 infections, the relevance of this small group was not significantly recognized. It was not until the identification of HPeV3 and its association with severe disease, that HPeV infections were considered relevant pathogens in young children. However, epidemiological and clinical data remained limited.

PICORNAVIRUSES

Picornaviruses are small non-enveloped viruses containing a single-stranded RNA with positive polarity. The Picornaviridae family is one of the largest RNA virus families and contains an array of pathogens that infect both humans and animals. At present the family is divided into 8 genera, but current proposals made by the International Committee on Taxonomy of Viruses (ICTV) would increase this to 11: Enterovirus, Parechovirus, Hepatovirus, Kobuvirus, Aphthovirus, Erbovirus, Teshovirus, Cardiovirus, Tremovirus, Sapelovirus and Senecavirus (Fig. 1) of which the Enterovirus, Hepatovirus, Kobuvirus and Parechovirus genera include several important human pathogens.

The Enterovirus genus contains over 100 types, those identified earliest being subdivided into polioviruses (PV; 3 types), Coxsackie A virus (CAV; 23 types) and Coxsackie B virus (CBV; 6 types), and echoviruses (28 types), based on their ability to replicate in human or primate cells, their infectivity and pathogenicity in different animal species and their antigenic differences (82). New types were later identified and numerically classified as enterovirus 68-102 (58,71-73). Advances in molecular techniques and the accumulation of sequence data allowed for a more precise classification based on molecular rather than phenotypic characteristics and four distinct human enterovirus (HEV) clusters were identified (30,75) later forming the basis for defining the species HEV-A, HEV-B, HEV-C (containing PV) and HEV-D (97). Enteroviruses isolated from animals were classified in four additional clusters. Similar studies showed rhinoviruses to be close relatives of the enteroviruses, and rhinoviruses were reclassified by the ICTV as 3 new species within the Enterovirus genus; Human Rhinovirus A to C.
Figure 1. Unrooted phylogenetic tree, showing the relationship between human parechoviruses and other Picornaviridae genera. The proposed genera Sapelovirus, Senecavirus and Tremovirus are given in italics. The Rhinovirus genus, proposed to be merged with Enterovirus, is encircled in a hatched line. The tree was constructed based on amino acid differences, using the neighbour joining method. The following nucleotide sequences were obtained from GenBank according to recent proposals by the ICTV. 

**Parechovirus:** (HPeV1 (S45208); HPeV1 BNI-788SI (EF051629); HPeV2 (AJ005695); HPeV3 A308-99 (AB084913) and Can82853-01 (AJ889918); HPeV4 K251176-02 (DQ315670) and T75-4077 (AM235750); HPeV5 CT86-6760 (AF055846) and T92-15 (AM235749); HPeV6 NII561-2000 (AB252582) and BNI-67/03 (EU024629); Ljungan virus 174F (AF327921), 87-012 (AF327920) and 145SL (AF327922);

**Enterovirus:** PV1 (V01149); PV2 (M12197); PV3 (K01392); CAV10 (AY421767); CAV16 (U05876); EV71 (U22521); CBV3 (M16572); CBV6 (AF114384); echovirus 30 (AF311938); echovirus 11 (AJ577589); CAV9 (D00627); CAV20 (AF465514); CAV24 (D90457); EV68 (AY426531); EV70 (D00820); Simian EV (SEV, NC003988); Rhinovirus 1B (HRV-1B, D00239); HRV 14 (K02121);

**Aphthovirus:** Foot and mouth disease virus-A (FMDV-A, NC011450); FMDV-O (AY686687); FMDV-SAT1 (NC011451); equine rhinitis A virus (ERAV, DQ272577);

**Cardiovirus:** Encephalomyocarditis virus (EMCV, X87335); Theilovirus (TMEV, NC001366);

**Hepatovirus:** Hepatitis A virus (HAV, AJ299464); Teschovirus: porcine teschovirus (PTV, NC003985);

**Erbovirus:** equine rhinitis B virus (ERBV, AF3612153);

**Kobuvirus:** Aichivirus (AIV, AB010145); Bovine kobuvirus (BKV, AB084788); Sapelovirus: Porcine EV (new proposed name: Avian sapelovirus, PEV/ASV, AF406813)

**Senecavirus:** Seneca Valley virus (SVV, DQ641257);

**Tremovirus:** avian encephalomyelitis-like virus (AEV, AY275539).
Enteroviruses and rhinoviruses are common human pathogens (21,54) and are responsible for a wide variety of diseases and clinical manifestations. Rhinoviruses are predominantly associated with the common cold, whereas enteroviruses have been associated with meningitis, myocarditis, and poliomyelitis. Poliomyelitis, caused by PV, is expected to be eradicated over the next few years due to efficient vaccination programs introduced by the World Health Organization (WHO) in 1988. However, no vaccines are available for other enteroviruses, and these still constitute a significant clinical problem. Although enteroviruses are transmitted via the fecal-oral route, gastrointestinal and respiratory symptoms are reported less frequently than the more severe symptoms (21).

HPeVs were previously classified as members of the *Enterovirus* genus. Together with Ljungan virus isolated from rodents, HPeVs form a separate genus, *Parechovirus*, within the family *Picornaviridae* (97). Ljungan virus was identified in 1999, during a search for an infectious agent that could be linked to myocarditis in humans. The virus was isolated from bank voles (*Clethrionomys glareolus*) and was most closely related to HPeVs (63).

Molecular techniques are now frequently used to identify and type different human picornaviruses from clinical samples. Typing of enteroviruses and parechoviruses is of great importance to elucidate the clinical and epidemiological characteristics of these viruses. With respect to the WHO poliovirus eradication campaign, it is essential to differentiate between PV and non-PV enteroviruses to ensure that wild type PVs or revertant PV vaccine strains responsible for vaccine derived poliomyelitis are not circulating in populations where PV has been successfully eradicated. Moreover, the use of molecular techniques allows the identification of new types or variants, in contrast to traditional typing methods such as serotyping, where the antisera used are only directed against known types.

To maintain consistency with the traditional typing of known HEVs, as well as HPeVs, molecular methods have been directed against the capsid region, in particular, the VP1 region (31,67,68,99).

The identification of new HEV and HPeV types has dramatically increased since the turn of the century. Molecular data are rapidly being generated and submitted to data banks, allowing for more precise classifications and reclassification of different viruses within new genera. This will increase our understanding of the virus diversity in relation to pathogenesis and evolution.
HPeVs have a single-stranded, positive-sense RNA genome of 7300 nucleotides which has a typical picornavirus organization (Fig. 2) (95,97). A 5’untranslated region (UTR) of around 700 nucleotides precedes an open reading frame of 2200 codons. This is followed by a 3’UTR (80 nucleotides) and a poly(A) tail. As in other picornaviruses, the open reading frame encodes structural proteins at its 5’end and nonstructural proteins downstream. Picornavirus polyproteins are cleaved by virus-encoded proteases to give precursors and the final proteins. In the case of HPeV, it seems likely that only one protease, 3Cpro, is involved in processing. In addition to 3Cpro, the functions of the picornavirus 3Dpol protein (the RNA-dependent RNA polymerase) and 3B protein (VPg, a protein primer of RNA replication) are well-documented. 2C is relatively well-conserved in picornaviruses and appears to function in RNA replication and possibly capsid assembly (49), but its modes of action are not fully understood. In HPeV, 2C appears to have ATP hydrolysis and AMP kinase activities (86), which may be involved in RNA replication. The proteins 2B and 3A are both
small proteins containing hydrophobic regions. They appear to interact with membranes and mediate cellular changes necessary for virus replication and release (45).

In addition to the proteins present in all picornaviruses, there are two loci which are highly diverse between different picornaviruses. These are the L and 2A regions (Fig. 2). The L region encodes a leader protein and occurs in only around half of picornavirus genera. Parechoviruses lack an L protein. Four different types of the 2A protein have been identified (Fig. 2). That in the Enterovirus (and probably Sapelovirus) genus, is a chymotrypsin-like protease involved in cleaving the polyprotein at its own N-terminus and also in host-cell protein synthesis shutoff (82). Although diverse in sequence, the 2A proteins of the Parechovirus, Kobuvirus and Tremovirus genera are related and share conserved motifs with a group of cellular proteins involved in the control of cell growth (26). The significance of this observation has not been established, but one function reported for the HPeV protein is RNA binding (85).

Parechoviruses, together with kobuviruses, have another major difference from other picornaviruses in that the structural protein VP0, usually a precursor of VP4 and VP2, is not cleaved, and so there are only three structural proteins rather than the four typically seen. As this VP0 maturation cleavage is thought to be critical for capsid stability and the acquisition of infectivity, this raises questions about these parameters in parechoviruses. Possibly other structural changes are involved in parechovirus maturation, but these remain obscure. Another distinct feature of kobuviruses and parechoviruses is the absence of VP0/VP4 myristoylation, a modification which is seen in most other picornaviruses (94).

**HISTORIC OVERVIEW OF ECHOVIRUSES 22 AND 23**

HPeVs were first isolated in 1956 by Wigand and Sabin (102) from children with diarrhea and were classified in the genus Enterovirus as echovirus 22 and echovirus 23. In this first report it was noted that these viruses were also identified in two patients with aseptic meningitis and three patients with febrile respiratory disease. When first isolated, they exhibited distinct growth characteristics from other enteroviruses, such as difficulty in adapting to monkey kidney cells. However, the cytopathogenic effect (CPE) seen in monkey kidney cells was generally similar to that seen with enteroviruses. Early reports identified echovirus 22 by cell culture or increase in neutralizing antibodies in children with gastrointestinal symptoms or respiratory infections (6,8,59). These associations were confirmed by WHO data from 1967 to
1974 showing that in patients with echovirus 22 infections, gastrointestinal symptoms and respiratory infections occurred at about the same frequencies (29% and 26%, respectively) while for HEVs these frequencies were much lower (9% and 15%, respectively). Central nervous system (CNS) symptoms were also reported but occurred less often, in 12% of the echovirus 22 infections, compared to 46% in other echovirus infections (21,39,96). Severe conditions associated with echovirus 22 infections, such as encephalitis, paralysis, and, myocarditis have been described occasionally (18,44,55,84). One report suggested an association with hemolytic uremic syndrome on the basis of 10 patients (74), and a publication from 1997 describing an outbreak of echovirus 22 infection in 19 neonatal intensive care unit patients suggested that for gastrointestinal disease with features of necrotizing enterocolitis, echovirus 22 infection should be considered (9).

A large Swedish study from 1993 retrospectively identified during a 25-year observation period (1966 to 1990) 109 patients with echovirus 22 infection. Clinical data were collected from 57 patients. Again, diarrhea was found most frequently, followed by respiratory infections. In 9% of the patients, encephalitis was clinically suspected, and one case of myocarditis was found (15). During the same study period, only five patients were identified with echovirus 23 infection, showing mild signs of gastroenteritis and/or respiratory infection (16). Until then, only one report had described echovirus 23 spread in a neonatal unit (5). Ehrnst et al. were the first to describe the specific epidemiological features of echovirus 22 and 23 infections (15,16). From these studies it was concluded that infections with echovirus 22 behaved differently from other HEV infections. Indeed, sequence analysis of the full-length genomes showed echovirus 22 and 23 to be distinct from other members within the *Enterovirus* genus (20,29), and further studies emphasized their characteristic molecular and biological properties (39,95,96). They were also genetically distinct from other picornavirus genera and consequently were renamed in 2000 human parechovirus 1 and 2 and classified as members of the human parechovirus species within a new genus *Parechovirus* (43).

**THE EXPANDING HUMAN PARECHOVIRUS SPECIES**

The establishment of a new genus within the picornavirus family was the focus of two key reviews describing the biology and clinical relevance of the new group of HPeVs, which contained two members named HPeV1 and -2 (39,96). From data available at that time, it was concluded that HPeV1 occurred frequently, predominantly in children with mild respiratory and
gastrointestinal symptoms. Occasionally, HPeV1 infection could give rise to severe symptoms such as myocarditis, encephalitis, pneumonia, meningitis, and flaccid paralysis. HPeV2 infections, however, appeared to be rare (39,96).

At the same time, molecular techniques were rapidly becoming state-of-the-art methodologies in many laboratories. The complete sequence of HPeV2 was published in 1998 by two independent groups (20,65). However, the genome of HPeV2 type CT86-6760 (65) appeared to be different from that of HPeV2 type Williamson, previously known as echovirus 23 (20).

In 2004, a third HPeV type was isolated in Japan by cell culture from a stool specimen of a 1-year-old child with transient paralysis. This new strain, designated A308/99, could not be neutralized with known antisera against human picornaviruses (including antisera against HPeV1 and HPeV2).

Shortly after the identification of HPeV3, a fourth HPeV type was identified in both The Netherlands (chapter 8) and the United Kingdom (3). In addition, phylogenetic analysis of all known HPeV types, including the second HPeV type 2 (CT86-6760) showed the CT86-6760 strain to be genetically distinct from the prototype HPeV2 strain, forming a fifth HPeV cluster along with 4 additional strains isolated in California between 1973 and 1992 (89). In 2007, a sixth HPeV type was identified following isolation from a child with Reye’s syndrome (101), and in 2008, 8 new HPeV types were genotypically characterized in Pakistan (HPeV7) (50), Brazil (HPeV8) (14), Thailand (Oberste et al, unpublished) and The Netherlands (HPeV14) (Chapter 5), thereby bringing the number of HPeV types to fourteen (Table 1).

Table 1 Prototype HPeV strains.

<table>
<thead>
<tr>
<th>Type</th>
<th>Strain</th>
<th>Origin</th>
<th>References</th>
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<tbody>
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<td>HPeV1</td>
<td>Harris/echovirus 22</td>
<td>Ohio, USA</td>
<td>(29,102)</td>
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<td>HPeV2</td>
<td>Williamson/echovirus 23</td>
<td>Ohio, USA</td>
<td>(20,102)</td>
</tr>
<tr>
<td>HPeV3</td>
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<td>Aichi, Japan</td>
<td>(31)</td>
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<tr>
<td>HPeV4</td>
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<td>(chapter 8)</td>
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<td>HPeV5</td>
<td>CT86-6760</td>
<td>Connecticut, USA</td>
<td>(65)</td>
</tr>
<tr>
<td></td>
<td>T92-15</td>
<td>California, USA</td>
<td>(3)</td>
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<tr>
<td>HPeV6</td>
<td>NII561-2000</td>
<td>Niigata, Japan</td>
<td>(101)</td>
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<td>(50)</td>
</tr>
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<td>(13)</td>
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<td>HPeV14</td>
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EPIDEMIOLOGY OF HPeV INFECTIONS

HPeV1 is a widespread pathogen that occurs globally, mainly infecting young children (38,39,96). Data reported to the U.S. National Enterovirus Surveillance System at the Centers for Disease Control and Prevention during 1983 to 2003 showed that HPeV1 accounted for 0.8% of the detected non-PV HEV and HPeVs in neonates and for 2.3% in the older age group. HPeV2 was not reported (42). In comparison, echovirus 30, one of the most common HEV serotypes, was detected in 6 to 12% of the HEVs isolated from neonates and older age groups. In a French surveillance over 2000 to 2004, HPeV1 was found in 0.6% of 2,757 patients reported with HEV or HPeVs (4). In contrast, a surveillance of HEV and HPeV during 1971 to 1992 in Finland reported that HPeV1 was one of the six most common HEVs and HPeVs and was isolated in 8% of the patients (24).

The idea that HPeV infections are frequent and widespread is illustrated by the high seroprevalences found in different parts of the world (1,38,59,88,96). In neonates, 95% had antibodies against HPeV1, evidently of maternal origin. At around 6 months of age, about 50% had antibodies, increasing rapidly to >90% in children >1 year of age (59,96). Among adults, seroprevalence for HPeV1 was 97%, while antibodies against echovirus 30 were found in 30% of the Finnish adults (38). In 219 pregnant mothers from children followed in a Finnish cohort study on type 1 diabetes, HPeV1 seroprevalence was 99% (98). In this prospective birth cohort study, HPeV1 antibody prevalence was 20% at 12 months and 72% at 24 months, which was lower than in the previous cross-sectional studies. At 36 months, almost all children (98%) in this study had antibodies against HPeV1. Therefore, from all studies it can be concluded that most individuals become infected with HPeV1 before adolescence. The seroprevalence for HPeV3 was found to be much lower, where HPeV3 seroprevalence had increased to 87%, above the age of 40 (31). Unfortunately, seroprevalence data on the newer HPeV types are lacking.

LABORATORY DIAGNOSIS OF HPeV INFECTION

The classical method for diagnosis of infection with HEVs or HPeVs has been virus isolation in cell culture from different clinical samples such as stool, throat swabs, cerebral spinal fluid (CSF), and blood. The standard cell culture for isolation of HEVs and HPeVs involves at least three cell lines, usually including monkey kidney cells and human fibroblasts. When a CPE is observed, the isolated virus can be identified by neutralization with a panel
of specific antibodies (including antisera against HPeV1 and -2). CPE produced by HPeV is not that different from that produced by HEV and HPeVs may therefore easily be identified as HEVs when specific serotyping is not routinely performed.

For detection of picornaviruses in stool samples and throat swabs, conventional cell culture is still widely used. However, reverse transcriptase PCR (RT-PCR) to detect HEV in CSF has shown to be faster and more sensitive than cell culture (80,81,100). Therefore, PCR is the preferred method for detection of viruses in CSF (17,77). RT-PCRs that detect HEV target the 5'UTR, which is highly conserved and therefore suitable to detect all HEV serotypes (7,68). Since the nucleotide sequences of the HPeVs are quite divergent from the HEVs, pan-EV RT-PCR fails to detect HPeVs (7,27,28,66). HPeV infections of the CNS will therefore be missed if only an HEV-specific RT-PCR is performed. Several conventional end point RT-PCR assays have been developed for detection of HPeV1 and -2 (48,66,77,90). Nowadays, in most diagnostic laboratories real-time PCR has become state-of-the-art. This testing method combines amplification by PCR with fluorescent probe detection of amplified product in a closed tube format, therefore eliminating the need of post-PCR analysis and decreasing contamination risk.

Detection of HEV and HPeV by real-time PCR is faster and less laborious than conventional cell culture or endpoint PCRs. For HEV it has been shown extensively that PCR is more sensitive than cell culture (17,81), not only for CSF but also for other clinical samples (2,91,100). For HPeVs, comparative studies have not been published yet. In a 10-month-old boy with encephalomyelitis, HPeV1 could be detected in CSF by PCR but not by cell culture (47), indicating that PCR is more sensitive.

RECEPTOR USAGE AND REPLICATION

The first HPeV sequence revealed the presence of an arginine-glycine-aspartic acid (RGD) motif close to the C-terminus of VP1 (Fig. 3). As this motif is found in a number of cellular and viral proteins which recognise integrin molecules, it suggests a role for the motif in the initial interaction with these cell surface receptors. All subsequent evidence confirms this initial supposition (11,40,94). The RGD motif, although in a relatively variable context, is itself absolutely conserved in HPeV1, -2, -4, -5, and -6 (Fig. 3). This motif has also been identified in two enteroviruses, CAV9 and echovirus 9 and also in FMDV, a member of the Aphthovirus genus (12,19,104). There is some conservation of flanking residues of the motif seen in these
picornaviruses and in HPeV, but while mutation and deletion studies show this region to be nonessential for their replication (23, 25, 83,105) deletion of the motif is lethal to HPeV1 (17).

Several papers have indicated that HPeV1 is recognised by integrins including αvβ1 and αvβ3, which recognize the RGD motif, and this interaction is followed by internalisation via endosomes (40,76,77,94). The motif was found to be essential for HPeV1 replication through mutation and deletion studies (17). Interestingly, the motif was found to be absent in HPeV3 to 14 (Fig. 3) and they are thought to enter the cell via an RGD-independent pathway.

Following release of the virus RNA into the cell cytoplasm, it is translated to give the polyprotein containing all the virus proteins. Picornaviruses use a cap-independent mechanism for initiation of translation, driven by an internal ribosome entry site (IRES) in the 5'UTR (Fig. 4) (60). Potentially this allows the majority of host cellular mRNA translation to be shut off, as this proceeds by a cap-dependent mechanism, although there is little evidence that HPeVs bring about shutoff (94). Following translation and processing of the polyprotein, the RNA genome is replicated through interactions within specific domains within the 5'UTR (60,61) and 3'UTR. The role of the latter region in picornaviruses has not been fully elucidated and has not been studied in HPeVs.
Another region of critical importance in RNA replication is the *cis*-acting replication element (CRE), the site of uridylation of VPg to give the primer required for RNA synthesis which is predicted to be in the VP0 gene in HPeVs (3).

**EVOLUTION**

HPeVs exhibit several unique molecular features but also attain features commonly found in other picornaviruses, making these viruses interesting in terms of evolutionary studies on the group itself and on their place in the picornavirus family.

RNA viruses are known to evolve rapidly within a population due to the genetic flexibility of the genome. Mutations, recombination, and segment reassortment all contribute to the genetic variation and evolution of RNA viruses and can result in a changed spread and pathogenicity within a population (41,57). The different genomic regions of picornaviruses each have different functions, which are reflected by their evolution. The protein capsid is under constant immune pressure, and in order to evade immunity the virus has to constantly change its appearance. Due to the gradual accumulation of mutations, the capsid region is known to be the most diverse region within the genome (92,93). The nonstructural region is driven by functional pressure, due to the functional requirements of the proteins.
encoded within this region. While phylogenetic analysis of the capsid region can distinguish the HEV types according to their classification, phylogenetic analysis of the nonstructural region of HEV shows inconsistent clustering of types. This was attributed to recombination between nonstructural regions of different types, rather than convergent evolution of the nonstructural region (92). Lukashev et al. recently proposed that HEV species can exist as a pool of a finite array of capsid genes and an infinite number of nonstructural genes which can freely evolve and recombine independently from another (52,53).

Recombination has been extensively studied in PVs (10,46,56,79), in particular, in vaccine-derived PV (13,22,37) and non-PV enteroviruses (51,53,64,69,70,87,92,93) and can have a profound impact on clinical outcome. As the HPeV group is a very small group containing only 14 members, studies on the evolution of the HPeV genome have been limited. With the identification of new HPeV types, recombination was suggested to play a role in the evolution of HPeVs (3,50,92,106, and chapter 8). Moreover, Simmonds et al. showed HPeVs to exhibit similar characteristics as other frequently recombining viruses (93).

**THESIS OUTLINE**

This aim of this thesis is to study the clinical and molecular characteristics of the HPeV group.

Part 1 points out the clinical relevance of HPeV infection in infants and the need for more specific diagnostics of both HPeV and enteroviruses. In chapter 2 the clinical symptoms associated with HPeV1 and HPeV3 infections are studied and it becomes apparent that HPeV infections are relevant pathogens in young children. A specific HPeV real time PCR to rapidly detect HPeV infection is described in chapter 3. This assay is used to retrospectively screen for HPeV in CSF and stool samples over a 3 year period (2004-2006) to establish the prevalence of HPeV infection among children (chapters 4 and 5). To determine the prevalence of the different HPeV types circulating, a method is developed to directly genotype HPeV from clinical material (chapter 5 and 7). In chapter 7 the detection of HPeV and HEV by PCR and genotyping is further evaluated in relation to culture characteristics and serotyping. The clinical manifestations of the newer HPeV types, HPeV4 to -6 are explained in chapter 6.
Part 2 describes the identification of new HPeV types and the likelihood of recombination among HPeV strains. Within this thesis, 2 new types are described (HPeV4 (chapter 8) and HPeV14 (chapter 5)). To study recombination among HPeV, two distant regions (VP1, used for typing, and 3Dpol, the polymerase nonstructural protein) are analysed to determine whether type-specific segregation observed within the capsid, is lost or carries over within the 3Dpol gene. The occurrence of recombination among HPeV is compared to that of HEV (chapter 9). These studies are extended with the generation and analysis of additional full length sequences of the predominantly circulating strains HPeV1 and -3 (chapter 10) to study diversity, dynamics and recombination breakpoints within the HPeV group.

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