Clinical and molecular insights into human parechovirus infection

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Human Parechoviruses (HPeV) have predominantly been associated with mild gastrointestinal and respiratory symptoms. Occasionally, more severe symptoms were described to be associated with HPeVs. Nonetheless, these viruses were considered irrelevant for routine diagnostics in comparison to human enterovirus (HEV) infection, which are the major viral cause of neonatal sepsis and meningitis. This thesis was started with a search for HPeVs in cell cultures, which lead to the finding that HPeV3 infections are associated with more severe disease than HPeV1 infections. From our studies it became clear that HPeV3 is clinically different from other HPeV infections. Not only do we show HPeV3 to be clinically different, but HPeV3 is also found to exhibit certain biological and molecular traits different from other HPeV types.

Chapter 1 is a general introduction to our current knowledge of HPeV infections. When first described in 1956, HPeV1 and -2 infections were considered mild. It took almost half a century, before a third HPeV type was described. Already this virus was molecularly distinct and was found to lack the critical epitope for integrin binding (Arginine-Glycine-Aspartic Acid, RGD). HPeVs exhibit a similar cytopathogenic effect (CPE) in cell culture as enteroviruses. As the 5'UTR of HPeV is different than that for enteroviruses, HEV specific PCRs will not detect HPeV infections, leading to a miss- or underdiagnoses. From 303 HEV positive cultures between 2000 and 2005, 37 cultures (12%) could be identified as either HPeV1 (n=27) or HPeV3 (n=10) (chapter 2). Clinical characteristics were scored by questionnaires and infections with HPeV3 were found to be significantly more often associated with severe symptoms and with a younger age in comparison to infections with HPeV1. We hypothesized that HPeV3 has a different cell tropism, in comparison to HPeV1, ruled by its absence of the RGD motif which could indicate an RGD independent cell entry mechanism.

To screen more efficiently for HPeV infections, we developed a rapid HPeV-specific real-time PCR with a specific primer pair and a single degenerate probe that could detect all known HPeV types at the time (n=6) (chapter 3). This method was evaluated for sensitivity and specificity by analysing all known HPeV types as well as other common human picornaviruses such as enteroviruses, rhinoviruses and hepatitis A virus. The assay reached a sensitivity of 75 copies per ml clinical sample, comparable to our HEV specific PCR, and was highly specific. Infections with HPeV are clinically indistinguishable from HEV infection. Since both viruses can be associated
with the same array of clinical symptoms, we introduced the HPeV real time assay in parallel to the HEV assay for diagnostic purposes. Retrospective screening using the newly developed PCRs on CSF samples from children <5 years lead to an increase of 31% more positive samples than when screened for HEV alone (chapter 4). The study involved 761 CSF samples obtained between 2004 and 2006. From the 761 CSF samples prospectively screened for HEV infection, 716 CSF samples were available for retrospective screening of HPeV infection. We found 33 HPeV infections (4.6%). In comparison, we found 108 HEV infections (14.1%). Children infected with HPeV were found to remain in hospital for a mean of 7 days of which 82% received antibiotics for 5.7 days as a result of a negative HEV infection diagnosed at the time. The study described in chapter 4 points out the clinical relevance of the implementation of the HPeV PCR and shows HPeV as a second viral cause for neonatal sepsis and meningitis. Adequate diagnostics is essential for good paediatric care and to avoid unnecessary hospital stays and treatment.

Within this study, HPeV prevalence varied over the years, with only one positive infection in 2005. In comparison, HEV infections of the central nervous system (CNS) remained constant over the years. That the variability might be due to a specific HPeV type exhibiting neurotropic characteristics was supported by our screening of stool samples, the same period (2004 to 2006), where stool obtained from children < 5 years were directly typed by PCR and sequencing (chapter 5). In 2005, the year we observed a low prevalence of HPeV in CSF, we did not identify any HPeV3 infections. Thus, these data supported our hypothesis that HPeV3 may infect different cell types, such as neural cells, with a more severe outcome.

Screening of stool samples obtained from children less than 5 years of age showed a high percentage of children to be positive for HPeV (n=225, 16.3%). This was comparable to the percentage HEV positive children (n=253, 18.4%). When all age groups were included, as in the analysis of stool samples from 2007 and 2008 (chapter 7), a lower prevalence was found for both HEV and HPeV. In these studies, HPeV1 was found to be the predominant strain, followed by HPeV3. HPeV4, -5, and -6 were identified less frequently, although the HPeV4 prevalence was quite high in 2007. To determine the clinical manifestations of the new HPeV types (4-6), questionnaires were used to score for different clinical parameters, such as age, hospital stay, and disease symptoms. Infection with HPeV4, -5, and -6 were found to be predominantly associated with mild symptoms (chapter 6) as seen for HPeV1 and -2. Only three children with HPeV4 infection were found to suffer from neonatal sepsis or meningitis.
By direct genotyping we have identified four strains that lacked the RGD motif. The strains could not be cultured. One strain could be typed as HPeV1 and the other three were characterized as HPeV5. These types should contain the RGD motif, but instead of the RGD motif at the 3’end, the strains contained a different specific consensus sequence at that site (chapter 5). The insertion of the specific sequence found in the four strains could indicate a second RGD-independent pathway.

A new HPeV type, which also could not be cultured, was identified by direct genotyping. The strain was designated HPeV14, since it was discovered after the identification of HPeV7 to -13. None of the new HPeV types (7 to 14) that were all identified by PCR were found to have the RGD motif. Interestingly, we found three HPeV1 strains to be most closely related to the Harris strain identified in 1956. The Harris strain was not identified in any of the other recent studies and was thought to cease circulation.

The value of direct stool screening and genotyping was assessed in chapter 7. A higher percentage of HPeV and HEV positive samples was identified by PCR in comparison to culture. This was found to be significantly correlated to the CT value in PCR. In addition more samples could be typed by genotyping rather than by serotyping. From the 6 cell lines used (HT29 (colon carcinoma), RD (rhabdomyosarcoma), Vero (African green monkey kidney), tMK (tertiary monkey kidney), Hel (human embryonic lung), and A549 (lung carcinoma)), both HPeVs and enteroviruses grew efficiently on the HT29 cell line. Specific types were found to grow exclusively on certain cell lines, such as Coxsackie A viruses on RD cells and HPeV3 on A549 and vero cells, supporting our hypothesis of a different cell tropism for HPeV3.

The second part of the thesis describes the identification and characterization of HPeV4 (chapter 8). The criteria for classification we used for this new HPeV genotype were based on those formulated by Oberste et al., (2004) for enteroviruses. Nowadays, those criteria are considered to be suitable to type HPeV strains. From this study, we describe for the first time the occurrence of recombination to play a role in HPeV evolution. To study this further, we compared two different genomic regions (VP1 and 3Dpol) from 37 isolates obtained between 2000 and 2006 (chapter 9). Recombination was frequently observed between types 1, 4, 5, and 6. The likelihood of recombination was found to be correlated to the VP1 divergence and difference in years of isolation between the strains, resembling that of enterovirus species B. The greater the divergence and temporal separation, the higher the likelihood of recombination was found to occur. Interestingly, no recombination was observed for HPeV3.
In chapter 10, we generated 18 additional full length sequences of the commonly circulating types 1 and 3. In total, we analyzed 35 full length sequences, including previously published sequences. As shown in the previous study (chapter 9), recombination was frequently observed for HPeV types 1, 4, 5, and 6. Recombination was also observed for HPeV3, indicating that HPeV3 recombination is possible. However, in comparison to the other HPeV types, this was observed to be much more restricted (chapter 10), possibly reflected by its different cell tropism, limiting co-infection to occur.

Sequence scans across the genome showed the major recombination breakpoints to flank the capsid region. Additional recombination sites were found more downstream for HPeV5 and -6. In contrast to previous studies depicting recombination among HPeV based on Bootscan which relies on these parental strains, not known for HPeV, our studies provide statistical support for the occurrence of recombination by comparison of the strains to one another and were able to depict recombination sites more precisely per type.

This analysis also showed a loss of perfect type-specific segregation within the capsid region that was linked to HPeV1. During our first study, we found the HPeV1 strains identified within our study to form a separate cluster from the HPeV1 strain isolated in 1956. However, only one Harris strain was known at the time. In chapter 10, we were able to analyze the diversification of the 2 clusters with two additional strains, most closely related to the Harris strain. Divergence analyses between and within the different type specific clades showed a distinct distribution of divergence and pair-wise distances of this group. The divergence between the two groups was found to be slightly lower than that found between other types, but greater than the divergence within other HPeV groups. The data provide statistical power to classify the HPeV1 group in at least two subtypes and suggests extension of the classification criteria (chapter 8) to include the characterization of highly diverse types.

In chapter 12, the results of this thesis are placed in perspective of our current knowledge of HPeV and a direction towards future research is given.