The 3-dimensional play of human parechovirus infection; Cell, virus and antibody

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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.
CHAPTER 5

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°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°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 μg/ml of streptomycin in 37°C humidified atmosphere with 5% CO2. 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 TCID50 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 CaCl2 and MgCl2) 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).](image)

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
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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Ethical approval: 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. The use of patient sera obtained for diagnostic purposes has been approved under the Research Code of the Academic Medical Center.
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