Temporal Relationship between Human Parechovirus 1 Infection and Otitis Media in Young Children

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Background. Human parechovirus (HPeV) 1 is a common virus that infects almost everyone during childhood. Because clinical symptoms are poorly documented, we evaluated the symptoms associated with HPeV1 infection in a cohort of children followed prospectively from birth at 3-month intervals.

Methods. Symptoms such as fever, cough, those of the common cold, otitis media, and gastroenteritis were determined from hospital records and from questionnaires administered to the parents of 59 children during regular study visits. HPeV1 infections were diagnosed by measuring neutralizing antibodies in follow-up serum samples. Additionally, HPeV RNA was analyzed in middle ear fluid (MEF) and nasopharyngeal aspirate samples from 33 patients with otitis media by reverse-transcription polymerase chain reaction.

Results. Otitis media showed a clear association with HPeV1 infection—it developed in 50% of the 3-month follow-up periods that yielded evidence for HPeV1 infection but in only 14% of the HPeV1-negative periods (odds ratio [OR], 6.14 [95% confidence interval {CI}, 2.75–13.77]). In children with recurring otitis media, MEF samples were positive for HPeV in 15% of episodes. Cough was also associated with HPeV1 infection, but this association was weaker (OR, 3.67 [95% CI, 1.66 – 8.09]). Other symptoms were not linked to HPeV1 infection.

Conclusions. HPeV1 infections are common in childhood and may cause otitis media and cough.

Human parechoviruses (HPeVs) have aroused growing interest in recent years. Although HPeV1 and HPeV2 (formerly known as echovirus 22 and 23) had been isolated by 1956 [1], other serotypes have been identified only recently. HPeV3 was isolated in 1999 [2–5], and now 6 types are known [6–8]. PeVs are small, positive-strand RNA viruses that belong to the Picornaviridae family. They had been classified as enteroviruses but were later separated to form a genus of their own, on the basis of genomic differences. In addition to the 6 HPeV types, a rodent virus, the Ljungan virus, belongs to the same genus.

HPeV1 is a common virus that infects mainly young children. Most HPeV1 infections occur before the age of 12–15 months [9–11] and are clinically mild or asymptomatic. HPeV1 infections have also been linked to gastrointestinal symptoms, particularly diarrhea, as well as to respiratory tract symptoms and to some more-serious infections in newborn infants [9, 12–18]. Almost all adults (99%) are seropositive for HPeV1 [11, 15].

This is the first study evaluating the clinical symptoms associated with HPeV1 infection in a healthy population prospectively observed from birth. Such a prospective design is optimal for the identification of symptoms associated with a specific pathogen. Another advantage is that these children represent a background population and were not selected according to any infectious symptoms. This sampling method is in contrast to that used in earlier studies, in which symptoms of HPeV1 infection have been looked for in hospitalized patients or in pa-
tients with infectious diseases, leading to a strong selection bias. This study is the first to provide evidence that HPeV1 infections commonly precede otitis media in children <2 years old. To study this association more closely, we also analyzed middle ear fluid (MEF) samples and nasopharyngeal aspirate (NPA) samples from children with recurrent otitis media.

METHODS

Study subjects. Our study population comprised 2 series. The first series included 59 children who were followed up prospectively and who represented the background population, and the second series included 33 children with recurrent otitis media. The prospectively observed children of series 1 took part in the Finnish Type 1 Diabetes Prediction and Prevention study; 22 (37%) of them were boys. These children were born in the university hospital of Turku or Tampere in Finland. They had an increased genetic risk for type 1 diabetes (they carried HLA-DQB1*02/*0302 or the *0302/x genotype, where x refers to alleles other than *02, *0301, or *0602) [19, 20]. The children were followed up from birth until the age of 9–42 months (mean, 21 months), and they visited their respective study centers at intervals of 3 months during the years 1995–2001. Clinical symptoms and medications were monitored by means of questionnaires completed by parents at each visit and hospital records. Blood samples were obtained at each visit, and cord blood samples were also available.

Series 2 included 33 children with otitis media (40 separate otitis media episodes) diagnosed and treated at Tampere University Hospital during the years 1996–1998. Samples were collected when tympanostomy tubes were placed. These children had had recurrent otitis media episodes (4–5 episodes per year) or otitis media secretoria. Their age ranged from 6 to 31 months (mean, 14 months), and 24 (73%) of the 33 children were boys. Altogether, 53 MEF samples and 13 NPA samples were collected. In 7 children, samples were obtained for multiple otitis media episodes (for 2 episodes in 6 children and for 3 episodes in 1). These children needed new tympanostomy tubes, and samples for recurring otitis media episodes were collected at the same time. MEF samples from both ears were available for 16 otitis media episodes and from only 1 ear for 21 episodes. In 3 cases, no MEF samples were obtained, and only NPA samples were available. In 10 cases, both MEF and NPA samples were obtained. The ethical committees of the participating university hospitals approved the study. Informed consent was obtained from the parents of the children studied.

Collection and analysis of clinical data (series 1). Clinical symptoms occurring after the previous study visit were recorded during the prospective observation period by the study nurse at each follow-up visit. In addition, clinical data were collected from hospital records for such children attending the hospital for the treatment of any disease.

The correlation between HPeV1 infection and clinical symptoms was analyzed for each 3-month interval between consecutive visits. The frequencies of various symptoms occurring during the same 3-month sampling interval as HPeV1 antibody seroconversion were compared with the frequencies during other sampling intervals to identify symptoms possibly associated with HPeV1 infection. For persistent symptoms, such as recurrent otitis media, only the first occurrence of symptoms was taken into consideration. In addition, the overall frequency of symptoms in children experiencing HPeV1 infection during the follow-up was compared with that in children who did not have any HPeV1 infection.

Plaque-neutralization assay (series 1). HPeV1 serotype-specific antibodies were measured in serum samples from the 59 children who were followed up prospectively, using a standard plaque-neutralization assay [11, 15]. The prototype HPeV1 virus strain (formerly echovirus 22) and the human lung carcinoma cell line (A549) were obtained from the American Type Culture Collection (ATCC). Different dilutions of serum were incubated with the virus (100 pfu) for 1 h at 36°C and then overnight at room temperature. The samples were then added onto A549 cell monolayers grown in 6-well plates (Nunclon; Nunc), and a viscoso carboxymethyl cellulose medium (0.5% in cell culture medium; Sigma) was added on top of the cells to avoid spreading of the virus in culture medium. Cells were further incubated at 36°C for 48 h. The amount of infectious virus was measured by counting the plaques; the reciprocal of the last serum dilution able to block virus infectivity by 80% was recorded as the titer of neutralizing antibodies. First, the last serum sample of the follow-up was analyzed using a low serum dilution (1:4). All serial serum samples from children who had positive titers in this primary antibody screening were further analyzed using serial 4-fold serum dilutions. A 4-fold or greater increase in antibody titers between 2 follow-up samples (reaching a titer of ≥16) was considered significant, indicating acute infection.

Reverse-transcription polymerase chain reaction (RT-PCR) analyses of otitis media samples (series 2). RNA was extracted from samples by means of the Qiagen Viral RNA Mini Kit (Qiagen). HPeV-specific RT-PCR was performed as described elsewhere [21] using primers K28 and K30, which detect all known HPeV serotypes. Altogether, 53 MEF and 13 NPA samples were analyzed. Positive PCR fragments were detected using a liquid hybridization test developed for this study. The hybridization was performed as for the enterovirus detection method described elsewhere [22]. The HPeV-specific probe used (5’-CAGGGACATGTTACACG-3’) was labeled with europium and measured by time-resolved fluorescence. All positive samples were retested, and only repeatedly positive samples were regarded as positive.

Statistical analysis. The χ2 test was used to analyze clinical symptoms possibly associated with HPeV1 infection. Differ-
ences were considered statistically significant at $P < .05$; 95% confidence intervals were calculated by the same method.

**RESULTS**

**Association between HPeV1 infection and clinical symptoms in the birth cohort series (series 1).** Of the children who were followed up prospectively, 36 (61%) became seropositive for HPeV1 during follow-up. The rate of seropositivity increased with age: at the age of 1 year, 27% of the children had HPeV1 antibodies, compared with 78% at the age of 2 years. Most infections occurred between the ages of 9 and 21 months. The rises in levels of neutralizing antibodies were clear, with titers $>64$ in all cases. Antibody levels remained elevated in later follow-up samples.

The frequency of various clinical symptoms occurring during the same 3-month interval as HPeV1 antibody seroconversion was compared with the frequency of symptoms during HPeV1-negative intervals. Clinical symptoms that coincided with HPeV1 seroconversion most frequently included otitis media (50%), common flulike symptoms (47%), cough (44%), fever (22%), and gastroenteritis (8%) (table 1). Otitis media and cough were significantly more frequent during HPeV1-positive than HPeV1-negative intervals (odds ratio, 6.14 and 3.67, respectively; $P < .001$ for both). A similar trend, although not statistically significant, was observed for fever and flulike symptoms, but gastroenteritis showed no association with HPeV1 infection (table 1). Cough was diagnosed 62 times and otitis media 54 times, with symptoms coinciding in 18 cases. On 5 occasions, all 3 (cough, otitis media, and HPeV1 infection) occurred during the same 3-month period. Other symptoms occurred only rarely during an HPeV1-positive interval (table 1), and 17% of the children reported no symptoms at all.

Altogether, 42 children experienced at least 1 otitis media episode during the entire observation period. There was a slight trend toward there being more otitis media episodes in boys than in girls (86% vs. 62%; difference not significant). Children with otitis media had more HPeV1 infections than did children who did not have otitis media (71% vs. 35%; $P = .022$). Children who had otitis media were more likely to have an HPeV1 infection during the same period (33%) than during periods when otitis media was not diagnosed (7.5%) ($P < .001$) (table 2). This association was seen both among boys and girls.

Almost all HPeV1 infections occurred before the age of 2 years (only 2 children remained seronegative at age 2.5 years). The possible role played by reinfections at an older age could not be analyzed, because the length of the follow-up was limited (the mean age at the end of follow-up was 21 months). Most children had their first otitis media episode before the age of 1 year. Recurring and prolonged otitis media events continued in some children until at least age 2 years.

**Detection of HPeV RNA in patients with otitis media (series 2).** Of the 40 separate otitis media episodes, findings were positive for HPeV RNA during 6 episodes (15%) in any sample type (table 3). HPeV RNA was detected in MEF samples during 4 episodes and in NPA samples during 2 episodes. Only 1 of these 6 cases occurred in a girl; the other 5 occurred in boys. Both ears were infected in 2 cases, and 1 ear was infected in the other 2 cases (in 1 case, a sample was available from only 1 ear). The ages of the children positive for HPeV varied from 6 to 9 months, with the exception of 1 child who was 2 years old. The RT-PCR assay did not quantify the amount of virus present.

**Seasonality of otitis media and HPeV1 infections.** In series 1 (the prospective cohort), HPeV1 infections peaked in late 1998, coinciding with a peak in otitis media (figure 1). Two additional peaks were seen for otitis media, the first in late 1997 to early 1998 and the second in late 1999 to early 2000. During both peaks, a smaller HPeV1 peak was observed.

**Table 2.** No. of human parechovirus (HPeV) 1 infections and otitis media episodes diagnosed within the same or different 3-month sampling interval in 59 children followed up from birth.

<table>
<thead>
<tr>
<th>Sampling interval</th>
<th>HPeV1 status</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Otitis media</td>
<td>18</td>
<td>36</td>
</tr>
<tr>
<td>Otitis media</td>
<td>18</td>
<td>221</td>
</tr>
<tr>
<td>All intervals</td>
<td>36</td>
<td>257</td>
</tr>
</tbody>
</table>

**NOTE.** There was a significant association between otitis media and HPeV1 infection in the same follow-up interval ($P < .001$).
In series 2, most of the MEF and NPA samples were collected at the end of 1996 (25 samples during 19 otitis episodes), and the rest were distributed evenly throughout 1997 and 1998. Almost all (7/8) HPeV-positive samples were obtained in September and November 1996.

DISCUSSION

This is the first study to show an association between HPeV1 infection and otitis media. HPeV1 is known to cause upper respiratory tract symptoms, but no connection with otitis media has been reported previously. Our patients also had cough during HPeV1 infection, but this association was weaker. Cough is common in patients with otitis media; therefore, its association with HPeV1 may be secondary in nature. In this study, otitis media and cough coincided on 18 occasions, but on only 5 occasions were both associated with an HPeV1 infection. Thus, the majority of children who had otitis media together with HPeV1 infection did not have a cough, suggesting an independent association between otitis media and HPeV1 infection. Boys had otitis media more often than girls (86% vs. 62%; difference not significant), but there was no difference by sex in the association between otitis media and HPeV1 infection.

Otitis media was studied more closely by analyzing HPeV RNA directly in MEF and NPA samples collected from patients with otitis media. Fifteen percent of otitis media episodes were HPeV RNA positive in either MEF or NPA samples (table 3) (11% of otitis media episodes were positive in MEF samples). Of the 2 sample types, MEF samples are probably a better indicator of a causal association with otitis media, although the value of NPA samples has not been systematically tested. In our study, the proportion of HPeV-positive samples was clearly higher than that observed previously when HPeVs were analyzed in patients with otitis media by use of both MEF and NPA samples and multiplex RT-PCR [23], which is known to detect enteroviruses, rhinoviruses, and PeVs in one assay. In that study, 1416 acute otitis media episodes in 611 children were analyzed, and HPeV was found in only 3 MEF samples and in 10 NPA samples. Altogether, this is <1% of all episodes, suggesting that HPeV infection is probably not a major cause of otitis media.

The reason for the discrepancy between those findings and our own remains hypothetical. One possibility is that the earlier study used a multiplex RT-PCR assay, which may have been less sensitive to HPeV than our single RT-PCR, which was optimized to detect the HPeV genome. Moreover, our liquid hybridization method may have further increased the sensitivity of the assay.

Table 3. Reverse-transcription polymerase chain reaction results for middle ear fluid (MEF) and nasopharyngeal aspirate (NPA) samples from patients with otitis media.

<table>
<thead>
<tr>
<th>Samples or episodes</th>
<th>HPeV positive, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples (n = 66)</td>
<td>8 (12)</td>
</tr>
<tr>
<td>MEF samples (n = 53)</td>
<td>6 (11)</td>
</tr>
<tr>
<td>NPA samples (n = 13)</td>
<td>2 (15)</td>
</tr>
<tr>
<td>Otitis media episodes*</td>
<td>6 (15)</td>
</tr>
</tbody>
</table>

NOTE. HPeV, human parechovirus.

* For some otitis media episodes, MEF samples from both ears and NPA samples were available.

In series 2, most of the MEF and NPA samples were collected at the end of 1996 (25 samples during 19 otitis episodes), and the rest were distributed evenly throughout 1997 and 1998. Almost all (7/8) HPeV-positive samples were obtained in September and November 1996.
One could also speculate as to whether an RT-PCR test can be too sensitive, detecting positivity that has no clinical relevance, but we do not know the quantity of HPeV1 needed to cause symptoms. In the present study, MEF samples were obtained from patients with recurrent otitis media or otitis media secretoria, and, given the results for series 1 (the birth cohort series), one could assume that the rate of HPeV1 positivity might actually be even higher during the acute phase of otitis media. We did not have MEF samples from such patients available for this study, and further studies are needed to address this question. Furthermore, a recent study found otitis media in 40% of patients (8/20) whose HPeV1 infection was diagnosed by RT-PCR analysis of NPA samples [24]. This finding resembles our observation, even though a specific relationship between HPeV infection and otitis media was not proposed in that study.

No association was observed between the estimated time of HPeV1 infection and gastroenteritis, fever, or common flu. It has previously been reported that HPeV1 infection causes gastrointestinal symptoms [13]. In that earlier study, neonatal infections were searched for, whereas most infections in the present study occurred between 9 and 21 months of age. This difference in the ages of the children studied may explain the absence of gastrointestinal symptoms in our series of patients.

Other clinical features associated with HPeV1 infection in our study were similar to those observed in other studies. Most HPeV1 infections occurred during winter (figure 1), and children were infected at an early age. In another study, we found markers of previous HPeV1 infection in 20% of children by the age of 12 months, 72% by 24 months, and 89% by 30 months [11]. The age distribution for HPeV1 infection was quite similar in the present study (27%, 78%, and 90%, respectively). It is possible that the virus strain used in the measurement of neutralizing antibodies has an effect on antibody titters. We used the well-characterized ATCC prototype strain, but we did not compare the abilities of different virus strains to bind neutralizing antibodies. However, the proportion of children who developed antibodies against this prototype strain was very high, suggesting that this virus strain worked quite well in this assay. This is also supported by the fact that the antibody titers reached fairly high levels and did not turn negative after seroconversion. In addition, our earlier study showed that HPeV positivity in stool samples correlated with the rise in antibody levels in serum [11].

Symptoms associated with HPeV1 infection have been studied previously only in hospitalized patients. A common belief has been that most HPeV1 infections are asymptomatic, because the frequency of infection in young children is very high. However, only 6 (17%) of the children followed up from birth in the prospective cohort of this study had no reported symptoms at the time of HPeV1 infection. Most likely the true proportion of subclinical HPeV1 infections is much higher, because the symptoms reported may occur in connection with almost any of the infectious agents frequently encountered during early childhood. In any case, this study suggests very strongly that an HPeV1 infection may cause otitis media and cough in children <2 years of age. Because the follow-up covered only this age group, we were not able to determine whether the same effect is found in older children. Almost all children acquired HPeV1 before the age of 3 years, and primary infections must be very rare after this age. However, this does not exclude the possibility that secondary infections may be related to the development of otitis media in older children, and this question should be evaluated in a separate study.

Otitis media may be caused by both viruses and bacteria, and mixed infections are also common. It is generally believed that the virus often causes the acute phase, which can be followed by bacterial invasion in some patients. We think that the clear temporal relationship between HPeV1 infection and acute otitis media, as observed in series 1, supports a causal relationship and a direct effect of HPeV1. However, this does not exclude the possibility that bacteria also contribute to pathogenesis, because HPeV1 may cause mucosal damage and increase the risk of bacterial invasion. In addition, it is quite unlikely that HPeV1 (or any other microbe) could cause otitis media in every infected person. Instead, the development of otitis media is probably regulated by anatomical, immunological, and other host factors, the interplay of which will determine whether the infection leads to otitis media.

In the last few years, many viruses have been linked to otitis media [23, 25–28], and this study adds HPeV1 to the list. It shows that the role played by HPeV1 in otitis media may be more important than previously assumed, and it may be a factor in up to 30%–40% of cases in young children. The association was evident both in the prospective birth cohort series and in the patients with otitis media, and it was further strengthened by the fact that the first cases of HPeV1 infection and otitis media appeared before the age of 12 months at the same times of the year, mostly during the same 3-month sampling intervals and in the same children. Interestingly, HPeV was detected in MEF samples in the otitis media series, although these patients had had recurrent otitis media episodes for several months. In light of the results of the birth cohort series, one could speculate that the rate of HPeV-positive MEF samples may be even higher in patients with acute otitis media. These findings imply that further studies are needed to determine the possible role played by HPeV1 in otitis media.

**Acknowledgments**

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