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Seppala, Elina

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Human enterovirus and rhinovirus infections are associated with otitis media in a prospective birth cohort study

Elina Seppälä[a,*], Saara Sillanpää[b,c], Noora Nurminen[a], Heini Huhtala[d], Jorma Toppari[e,f], Jorma Ilonen[g], Riitta Veijola[h], Mikael Knip[i,j,k,l], Markku Sipilä[c], Jussi Laranne[c], Sami Oikarinen[a], Heikki Hyöty[a,m]

[a] School of Medicine, University of Tampere, Lääkärinkatu 1, FIN-33520, Tampere, Finland
[b] Department of Anatomy, School of Medicine, University of Tampere, Finland
[c] Department of Otorhinolaryngology, Head and Neck Surgery, Tampere University Hospital and School of Medicine, University of Tampere, Finland
[d] School of Health Sciences, University of Tampere, Tampere, Finland
[e] Department of Physiology, University of Turku, Turku, Finland
[f] Department of Pediatrics, Turku University Hospital, Turku, Finland
[g] Immunogenetics Laboratory, University of Turku and Turku University Hospital, Turku, Finland
[h] Department of Pediatrics, PEDEGO Research Unit, Medical Research Center, Oulu University Hospital and University of Oulu, Oulu, Finland
[i] Children's Hospital, University of Helsinki and Helsinki University Hospital, Helsinki, Finland
[j] Research Programs Unit, Diabetes and Obesity, University of Helsinki, Helsinki, Finland
[k] Folkhälsan Research Center, Helsinki, Finland
[l] Tampere Center for Child Health Research, Tampere University Hospital, Tampere, Finland
[m] Fimlab Laboratories, Pirkanmaa Hospital District, Tampere, Finland

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ABSTRACT

Background: Human enteroviruses (HEVs) and rhinoviruses (HRVs) have been linked to acute otitis media (AOM).
Objectives: The present study evaluates the aforementioned association in a birth cohort setting.
Study design: The cohort included 286 healthy infants (191 boys) followed from birth up to the age of 2 years in the Type 1 Diabetes Prediction and Prevention study in Finland. Stool samples were collected monthly and analyzed for the presence of HRV and HEV RNA using RT-PCR. Clinical symptoms were recorded by a questionnaire every 3–6 months.
Results: Altogether 610 AOM episodes were reported during the follow-up. 9.8% of the stool samples were positive for HRV and 6.8% for HEV. HRV positivity peaked at the age of 3–6 months declining gradually after this age, whereas HEV positivity peaked later, at the age of 12–24 months. The risk of AOM was increased in children who were HEV positive at least once at the age of 6–12 months (OR 2.2 [95%CI 1.1–4.2], $P = 0.023$) or who were HRV positive at least once at the age of 18–24 months (OR 2.3 [95%CI 1.0–5.2], $P = 0.042$). Having an older sibling, short breast-feeding and maternal smoking during pregnancy were also significantly associated with AOM.
Conclusions: HRV and HEV infections are frequent during the first months of life. The observed trend for increased risk of AOM in HRV and HEV positive children is in line with the results from hospital series suggesting that these viruses may play an independent role in the pathogenesis of AOM.

1. Background

Human rhinoviruses (HRV) and human enteroviruses (HEV) are RNA viruses which belong to the *Enterovirus* genus of the *Picornaviridae* family. HRVs, classified in three groups of HRV-A, HRV-B and HRV-C, are the most common viruses causing upper respiratory tract infections (URI) in man, and they have also been linked to lower respiratory tract infections, particularly in infants and elderly patients as well as in patients with asthma or immunodeficiencies. HEVs are classified in four groups (HEV-A to HEV-D) and are also a common cause of respiratory infections [1–3]. Both HRVs and HEVs have been linked with acute otitis media (AOM) in several studies. In one Finnish study, picornaviruses, mainly HRVs and HEVs,

* Corresponding author at: School of Medicine, University of Tampere, Lääkärinkatu 1, FIN-33520, Tampere, Finland.
E-mail address: seppala.elina.m@student.uta.fi (E. Seppälä).

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accounted for 60% of all the viral findings in children with AOM [4]. HRVs were detected in 24–41% of middle ear or nasopharyngeal specimens in children with AOM, whereas enteroviruses were found in 25% of these samples [5,6]. In a recent study by Chonmaitree et al. AOM occurred in about one-third of those children with URI caused by rhino- and enteroviruses [7]. However, the clinical relevance of virus-positive PCR-findings has been questioned because of the high sensitivity of PCR methods and because these viruses have also been frequently found in asymptomatic children [8,9]. Thus, it would be important to carry out studies where the overall frequency of HRV and HEV infections is evaluated in children who develop AOM and control children who do not. Such studies would not suffer from selection bias, which is typical for hospital series and would help to generate information about the etiological fraction of these viruses in AOM.

2. Objectives

The aim of this study was to evaluate the epidemiology of HRVs and HEVs in a cohort of children who were followed from birth to the age of 2 years. Stool samples were collected regularly from the children and samples were analyzed for the presence of HRVs and HEVs using sensitive RT-PCR methods. AOM episodes were recorded during the follow-up, which made it possible to correlate virus-positive episodes to AOM episodes in an unbiased way.

3. Study design

3.1. Subjects

The study population comprised 286 healthy infants, 191 boys and 95 girls, who were recruited to the prospective Type 1 Diabetes Prediction and Prevention study (DIPP) at Tampere University Hospital (n = 105) and Turku University Hospital (n = 181) in Finland. According to the DIPP study protocol all newborn infants whose families gave informed consent to the study were first screened using cord blood for HLA-DQ conferring susceptibility to type 1 diabetes, and those with increased risk were recruited to prospective follow-up to detect the possible appearance of diabetes-associated autoantibodies. The children carried the DQB1*02/DQB1*0302 or the DQB1*0302/*x* genotype, where *x* refers to alleles other than *02, *0301, or *0602/03 [10,11]. The children received vaccinations, including the inactivated polio vaccine (IPV), according to the National Immunization Program at well-baby clinics. The Ethics committees of participating university hospitals approved the protocol of the DIPP study. A written informed consent was obtained from the parents of all study children.

3.2. Recording of AOM episodes

The study subjects living in the Tampere University Hospital area visited the DIPP study center at the age of 3, 6, 12, 18 and 24 months, and the subjects living in the Turku University Hospital at the ages of 3, 6, 9, 12, 15, 18, 21 and 24 months. The children in the current study had altogether 1876 visits to the DIPP study clinics. During each visit a study nurse interviewed the parents using a standard questionnaire. The number of AOM episodes, which the child had encountered since their last visit to the clinic, was recorded. AOM included all parent-reported otitis media episodes that had been diagnosed by a physician (prolonged OM such as otitis media with effusion was counted only once when first diagnosed in the acute phase). Information about the diet and the type of daycare (care at home, family care, day care center, other) was also documented in the context of each visit. The number of siblings was recorded as well. In Tampere, information about mothers' smoking during pregnancy was gathered from the medical birth register in Finland. In Turku, this information was acquired through parental interviews.

3.3. Collection and preparation of stool samples

Parents collected stool samples from the study subjects at home every month and sent them to the laboratory by mail. We have previously shown that viral RNA preserves well in such shipment conditions [12]. The samples were stored at −20 °C or −70 °C until analyzed. The collection of stool samples was started at the age of 3 months.

3.4. Detection of rhino-and enteroviruses

Total RNA was extracted from 140 μl of 10% stool suspension in HANKS solution using QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. HEV and HRV RNA was amplified using reverse transcriptase (RT) reaction followed by PCR as described by Lönnrot et al. The PCR amplicons were detected by liquid-phase hybridization assay based on probes labeled with lanthanide chelates of europium and samarium [13]. This method allows the specific detection of HEVs and HRVs. We and others have previously shown that both HEV and HRVs can be frequently detected from stools using these methods [13–15]. The probes used in this assay differentiate HEVs from HRVs very well [12], but it is possible that the HRV probe does not bind well to all HRV C species [16].

3.5. Statistical methods

Binary logistic regression model was used as a multivariable analysis comparing the association of HRV and HEV positivity in stool samples with AOM episodes, taking into account the smoking habits of the mother during pregnancy as a confounding factor. Fisher's exact test was used to analyze differences between two groups of children: those who had not experienced any AOM episodes during the follow-up period vs. those who had encountered at least one AOM episode. The characteristics analyzed included gender, number of siblings, autoantibody status, starting age of daycare, duration of breastfeeding and maternal smoking habits during pregnancy. Mann-Whitney *U* test was used to analyze the effect of the factors mentioned above to the amount of AOM episodes recorded during the follow-up period. The software package used was SPSS, version 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Differences were considered significant at $P < 0.05$.

4. Results

The basic demographics of the children are shown in Table 1. The total duration of follow-up was 566 child-years and the median duration 24.2 months. Altogether 610 AOM episodes (1.1 episodes/child-year) were recorded. When analyzing AOM as a categorized variable (no AOM versus at least 1 AOM episode during the follow-up), having an older sibling was statistically significantly associated with AOM. As shown in Table 2, having an older sibling or a short period of exclusive breastfeeding increased significantly the average number of AOM episodes during the study period. In addition, children of mothers who smoked during pregnancy tended to have more AOM episodes compared to children of nonsmoking mothers. Other factors such as sex, the starting age of daycare and islet autoantibody status (used for the identification of children with increased risk for type 1 diabetes) were not associated with the frequency of AOM episodes (Table 3).

Table 1
Demographic characteristics of the study subjects (N = 286).

	Data available	n (%)	No AOM n = 82		≥1 AOM n = 204		*P*
			n	%	n	%	
Median duration of follow-up (mo)			24.1		24.2		0.488
Islet autoantibody-positive	286	82 (28.7)	24	29.3	58	28.4	0.886
First-born subjects	286	147 (51.4)	50	70.0	97	47.5	0.040
Daycare at the age of 12 mo	256	21 (9.0)	6.0	7.3	15	7.4	0.797
Exclusively breastfed at the age of 3 mo	255	220 (86.3)	63	85.1	157	86.7	0.735
Breastfed at the age of 6 mo	273	175 (64.1)	56	68.3	119	58.3	0.214
Mother smoked during pregnancy	265	17 (6.4)	3.0	3.7	14	6.7	0.410

Table 2
The association between different background factors and the frequency of AOM episodes.

		No. of AOM		
		Mean	Median	*P*-value[a]
Sex	Boy	2.21	2	0.347
	Girl	1.97	1	
Mother smoked during pregnancy	Yes	3.24	4	0.048
	No	2.04	1	
Duration of breastfeeding ≥6 mo	Yes	2.01	1	0.067
	No	2.35	2	
Exclusive breastfeeding at the age of 3 mo	Yes	1.75	1	0.006
	No	2.41	2	
First-born child	Yes	1.73	1	0.003
	No	2.55	2	
In daycare at the age of 12 mo	Yes	2.29	1	0.993
	No	2.22	2	
In daycare at the age of 18 mo	Yes	2.52	2	0.257
	No	2.11	1	
In daycare at the age of 24 mo	Yes	2.45	2	0.393
	No	2.19	2	
Islet autoantibodies	Yes	2.13	1	0.821
	No	2.13	1	

[a] Mann-Whitney *U* test.

Table 3
Proportion (%) of virus-positive stool samples during the follow-up (3–24 months of age).

		HRV positivity			HEV positivity			HRV and/or HEV positivity		
		Mean	Median	*P*	Mean	Median	*P*	Mean	Median	*P*
Sex	Boy	11.8	7.1	0.912	5.7	0	0.545	17.5	14.3	0.928
	Girl	10.5	7.1		7.4	0		18.0	14.3	
Mother smoked during pregnancy	Yes	10.3	5.6	0.970	7.7	5.6	0.189	18.0	15.0	0.405
	No	11.1	7.1		6.2	0		17.3	12.5	
Duration of breast-feeding ≥6 mo	Yes	11.8	8.3	0.121	6.5	0	0.766	18.0	14.3	0.351
	No	9.8	5.0		6.4	0		16.2	11.5	
Exclusive breastfeeding at the age of 3 mo	Yes	11.6	9.1	0.419	6.5	0	0.424	18.1	14.3	0.466
	No	11.2	5.9		5.8	0		17.0	11.8	
First-born child	Yes	7.8	5.0	<0.001	4.6	0	0.012	12.4	9.5	<0.001
	No	15.1	10.0		8.1	0		23.3	20.0	
In daycare at the age of 12 mo	Yes	13.1	8.3	0.730	6.3	0	0.607	19.4	13.3	0.998
	No	10.9	7.1		6.3	0		17.2	14.3	
Autoantibodies	Yes	11.7	8.4	0.937	6.7	0	0.887	18.5	14.3	0.706
	No	11.2	7.1		6.1	0		17.3	13.3	

Altogether 3,438 stool samples were collected (mean 12 samples per child), and 337 (9.8%) of the samples were positive for HRV and 234 (6.8%) for HEV. The proportion of HRV- positive stool samples was highest during the age of 3–6 months declining thereafter towards the age of 24 months, while the proportion of HEV-positive stool samples peaked later, at the age of 12–24 months (Fig. 1). Having an older sibling increased the rate of positivity for both HEV and HRV, while day-care and breast-feeding showed no such association (Table 3).

The detection of HEV was associated with the diagnosis of AOM episode in the same follow-up visit interval in children aged from 6 to12 months (OR 2.2 [95% CI 1.1–2.4], *P* = 0.023) (Table 4). Detection of HRV was also associated with the diagnosis of AOM in the same follow-up interval in children aged from 18 to 24 months (OR 2.3 [95% CI 1.0–5.2], *P* = 0.042). These trends remained, even if not statistically significant, when adjusted for maternal smoking during pregnancy and having older siblings (Table 4).

5. Discussion

The present study confirms previous findings showing a risk association between HRV and HEV infections and AOM [4–7]. Since

Table 4
Association of OM (≥1) with virus-positivity (≥1 one virus-positive stool sample during the defined age period) compared to virus-negativity (univariate and adjusted analysis).

Age period	& virus	No OM	≥1 OM		Adjusted by mother's smoking and having older siblings
		n (%)	n (%)	OR (95% CI)	OR (95% CI)
3–6 mo		N = 235	N = 28		
	HRV	89 (37.9)	15 (53.6)	1.9 (0.9–4.2)	1.7 (0.7–4.0)
	HEV	22 (9.4)	2 (7.1)	0.8 (0.2–3.4)	0.9 (0.2–4.1)
	HRV and/or HEV	99 (42.1)	15 (53.6)	1.6 (0.7–3.5)	1.3 (0.6–3.2)
6–12 mo		N = 145	N = 116		
	HRV	46 (31.7)	35 (30.2)	0.9 (0.6–1.6)	0.8 (0.5–1.4)
	HEV	17 (11.7)	26 (22.4)	2.2 (1.1–4.2)	1.8 (0.9–3.5)
	HRV and/or HEV	56 (38.6)	52 (44.8)	1.3 (0.8–2.1)	1.1 (0.6–1.8)
12–18 mo		N = 129	N = 78		
	HRV	39 (30.2)	18 (23.1)	0.7 (0.4–1.3)	0.6 (0.3–1.3)
	HEV	30 (23.2)	21 (26.9)	1.2 (0.6–2.3)	1.1 (0.6–2.3)
	HRV and/or HEV	58 (45.0)	34 (43.6)	1.0 (0.5–1.7)	0.8 (0.5–1.5)
18–24 mo		N = 96	N = 64		
	HRV	13 (13.5)	17 (26.6)	2.3 (1.0–5.2)	1.8 (0.8–4.3)
	HEV	23 (24.0)	16 (25.0)	1.1 (0.5–2.2)	0.9 (0.4–2.0)
	HRV and/or HEV	33 (34.4)	30 (46.9)	1.7 (0.9–3.2)	1.4 (0.7–2.9)

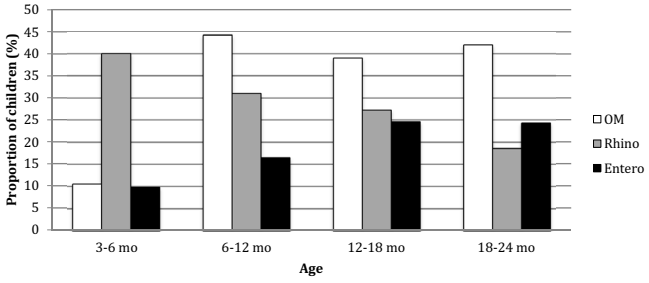

Fig. 1. Proportion of subjects with ≥1 episode of AOM, ≥1 positive rhinovirus sample and ≥1 positive enterovirus sample according to age.

the present study was based on a prospective follow-up of children who were not hospitalized or otherwise selected based on symptoms of respiratory infections or AOM, the study was not biased by factors that could lead to an overestimation of the role of these viruses in AOM. In addition, this study design made it possible to take into account the high frequency of these viruses in asymptomatic children including those who did not develop AOM. We indeed found that the frequency of these viruses was high also in children who did not report AOM. In addition, having older siblings seems to be an important risk factor since it doubled the amount of HRVs and HEVs in the child and significantly increased also the risk of AOM.

The results support the concept that these viruses can contribute to the development of AOM. However, the statistical significance of the observed associations was relatively weak and disappear when corrected by the number of comparisons (Bonferoni's correction) indicating that these findings should be confirmed in further studies in similar pediatric cohorts. This is important also because the proportion of AOM cases attributable to HRV and HEV infections may depend on age, and the circulation of these viruses in the given population.

In the current study the youngest children (age of 3–6 months) were most frequently HRV positive. This finding is in agreement with recent studies showing that HRV infections occur very early in life, the mean age of the first symptomatic HRV infection being 4–6 months [17,18]. In one study carried out in Finland, approximately 30% of the 6–24 month-old subjects with HRV present in their nasopharynx developed AOM within 4 weeks after URI onset [19]. In our study the association between HRVs and AOM episodes was not seen until the age of 18–24 months. The reason for the lack of this association in younger children is not known. Theoretically, it could be at least partly explained by viral-bacterial interactions in the nasopharynx. Multiple studies have shown that there is a direct relationship between the frequency of nasopharyngeal colonization by potential pathogenic respiratory bacteria (*Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*) and frequency of AOM. Two studies conducted by Faden et al. demonstrated the increase of colonization rates of these bacteria with age, the rates for *M. catarrhalis* being 26% at the age of 6 months, 72% at the age of 12 months and 77% at the age of 24 months. This finding supports many other studies concluding that bacterial colonization increases gradually and peaks at 2–3 years of age. These pathogenic bacteria may not cause symptoms until viral URI causes changes in the nasopharynx by inducing inflammation, changes in bacterial adherence properties and colonization, and Eustachian tube dysfunction [20–23].

In addition, there are many other factors influencing the pathogenesis of AOM. Maternal IgA and several other substances in breast milk protect breastfed infants from AOM and many other infections [24]. The preventive effect of breastfeeding has been documented in meta-analyses conducted by Uhari et al. and Bowatte et al. [25,26]. According to the latter study, breastfeeding appears to protect against AOM until the age of 2 years. The protective effect was observed to depend on the exclusiveness and duration of breastfeeding: exclusive breastfeeding for the first 6 months was associated with the strongest beneficial effect.

HEV infections peaked at the age of 12–24 months, at a slightly later age compared to that found in a surveillance study conducted in France during 2000–2004 [1]. This is in line with previous studies suggesting that HEV infections are less common in Finland than in most other European countries [27]. An association between HEV positivity of stool samples and AOM was seen at the age of 6–12 months, when AOM starts to peak.

According to previous studies approximately 70% of children experience at least one OM episode by the age of 24 months, with a peak incidence between 6 and 18 months and the incidence of AOM being 0.9–1.2 AOM episodes per child-year [28–31]. Our findings are in line with these previous observations. Several risk factors for AOM have been identified. Male gender and older siblings have been identified as some of the strongest risk factors associated with AOM [32,33]. Our finding that having older siblings increased the incidence of AOM is well in line with these previous studies. In our

study, the average number of AOM episodes encountered during the follow-up was slightly higher among boys than girls, but the difference was not statistically significant. Being in daycare at the age of 12 months did not have a significant impact on the amount of AOM episodes in our study. In addition, maternal smoking has been reported to be associated with AOM, and this trend was also seen in the present study. It should be noted that the trend for risk association of HRV and HEVs with AOM remained even when adjusted for mother's smoking and having older siblings.

Stool sample is a classical sample type used for the diagnosis of HEV infections. In addition, it has been recently reported that also HRV can be detected in feces during HRV infection in young children [14,34]. It was also recently observed that HRVs and HEVs can be detected simultaneously in middle ear and fecal specimens during AOM (Sillanpää et al., unpublished). In fact, HEVs and HRVs are detected in almost identical frequency in respiratory and stool specimen [35]. Hence, we used the positive stool sample as a marker of both HRV and HEV infection. However, one of the limitations of the present study is that respiratory samples were not collected for virus analyses. Further studies using a similar prospective cohort design accompanied by respiratory sample collection would be helpful to get more information about the causative role of HRVs and HEVs in the pathogenesis of AOM.

One of the strengths of this study was the unique study design, which was based on an unbiased birth cohort study, in which the temporal relationship of virus positivity of stool samples and AOM episodes could be assessed. The AOM diagnosis was always made by a doctor, and parents reported it on the regular visits. As already noted, the clinical relevance of virus-positive PCR-findings has been questioned because of the high sensitivity of PCR methods and high frequency of viruses in asymptomatic children. Such asymptomatic HEV and HRV infections are common and the rate of virus positivity in AOM patients should therefore be compared to that in matched control children who do not have AOM [8,9]. Shedding of HRV from the nasopharynx may also continue quite long, up to 5–6 weeks after the onset of the infection, and that of HEVs for up to 2–3 weeks from the nasopharynx and many weeks to months in the feces [8]. In our prospective follow-up study, the relatively long excretion is actually an advantage, since it enabled the detection these infections using samples, which have been collected regularly (monthly), irrespectively of the symptoms of acute infections. Thus, the fact that we found an association between AOM episodes and virus-positivity in this kind of case-control comparison where the time-dependences of these two events could be evaluated during prospective follow-up of children, support the role of HEVs and HRVs in the pathogenesis of AOM. However, the study focused on HEVs and HRVs, and further studies would be needed to evaluate their etiologic fraction in relation to other viruses previously linked to AOM.

As a viral infection often initiates bacterial AOM and viruses alone can also cause AOM, it would be beneficial to prevent the initial viral infection. This is demonstrated by the fact that influenza vaccine has decreased AOM episodes during influenza seasons by 32–71% [36,37]. The findings of this study and previous reports suggest that picornaviruses should be considered as targets when aiming at the prevention of AOM in children. Our previous study suggested that the oral polio vaccine (OPV), a live attenuated enterovirus vaccine, may considerably reduce OM episodes possibly by interfering with these other picornaviruses which cause AOM [38]. Thus, antiviral strategies targeting HRVs and HEVs may lead to a substantial reduction of AOM incidence in young children.

6. Conclusions

The present study, which was based on a unique population–based approach, supports previous observations, suggesting that both HRVs and HEVs contribute to the pathogenesis of AOM in young children. This emphasizes the potential of antiviral strategies in the prevention of AOM in this age group.

Conflict of interest

The authors declares that there is no conflict of interest.

Competing interests

Dr Heikki Hyöty and Mikael Knip are minor shareholders (<5%) and members of the board of Vactech LTD, which develops vaccines against picornaviruses.

Ethical approval

The Ethics committees of participating university hospitals approved the protocol of the DIPP study (decisions 228/94 in Turku and 97193 M in Tampere).

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