Human bocavirus in the nasopharynx of otitis-prone children

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ABSTRACT

Objectives: Human bocavirus (HBoV) is frequently identified in children with respiratory tract infections, and its role in acute otitis media (AOM) has been suggested. The disease associations for the closely related bocaviruses HBoV2-4 remain unknown. Increasing evidence shows that probiotics may reduce the risk of AOM of viral origin. Objectives of the study was to examine the prevalence and persistence of bocaviruses in consecutive nasopharyngeal samples (NPS) of otitis-prone children, and whether an association exists between HBoV and the child's characteristics, respiratory symptoms, and AOM pathogens, and whether probiotics reduce the occurrence of HBoV.
Methods: In a double-blind, placebo-controlled, randomized, 6-month intervention study, 269 otitis-prone children (aged 9 months to 5.6 years), consumed daily either one capsule of probiotics (*Lactobacillus rhamnosus* GG, *L. rhamnosus* Lc705, *Bifidobacterium breve* 99 and *Propionibacterium freudenreichii* JS) or placebo. After a clinical examination and NPS collected at three-time points, the presence and persistence of HBoV1-4 DNA in NPS was determined by RT-qPCR at the baseline, after 3, and 6 months.
Results: A high load (>10,000 copies/ml) of HBoV DNA was detected in 26 (17.1%) of 152 children, and 16 (10.5%) showed a prolonged presence of HBoV for at least 3 months. None had DNA of HBoV2-4. Higher number of siblings associated with increased HBoV prevalence ($p = 0.029$). Prevalence or persistence of HBoV was not significantly associated with other characteristics, respiratory symptoms, or AOM pathogens. Probiotic intervention significantly reduced the number of HBoV DNA-positive samples (probiotic vs. placebo: 6.4% vs. 19.0%, OR = 0.25, CI 95% = 0.07–0.94, $p = 0.039$).
Conclusions: HBoV, but not HBoV2-4, DNA occurs often in the nasopharynx of otitis-prone children, and may persist for 3–6 months. Probiotic treatment possibly reduced the presence of HBoV.

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1. Introduction

One of the most common infectious diseases among children is acute otitis media (AOM). Several clinical and epidemiological studies demonstrate a close association between AOM and respiratory tract infections (RTIs) of viral origin [1–3]. Especially rhinoviruses, enteroviruses, adenoviruses, and the respiratory syncytial virus have frequently been detectable in AOM cases [4–6].

Recent developments in molecular biology techniques and their adaptation for virology have led to the discovery of novel viruses. These include the human metapneumovirus [7], several human coronaviruses (e.g. HKU1, SARS) [8–10], and the human boca-

viruses (HBoV1-4) [11–13]. Since the discovery of HBoV in 2005, it has been frequently detectable worldwide, mainly in the respiratory tracts of young children [14]. HBoV2-4 primarily occur in stool samples, with HBoV2 seemingly associated with gastro-enteritis [11]. The prevalence of HBoV in the upper airways ranges from 1.5% to 19%, with frequent high rates of coinfections with other viral agents [12,15–20]. HBoV has been associated with upper and lower respiratory tract infections in children [17–19,21–24]. HBoV DNA also occurs in the nasopharynx, in middle ear fluids, and in serum of children with AOM [15,25–27], and HBoV infection may worsen clinical symptoms and prolong the clinical outcome of AOM [25]. Further studies to establish the causative role of HBoV in the development of AOM are, however, necessary.

Antibiotic treatment of recurrent AOM may lead to the antibiotic resistance of pathogenic bacteria, disturbances in the balance of the normal nasopharyngeal microbial flora, which

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promote colonization of AOM pathogens [28,29]. Probiotic bacteria offer an attractive option for re-establishing this microbial equilibrium and preventing infectious diseases. *Lactobacillus rhamnosus* GG in particular has been effective in the prevention of upper respiratory tract infections and in reducing the risk of acute RTIs in children attending daycare [30,31]. In otitis-prone children, a probiotic combination of *L. rhamnosus* GG, *L. rhamnosus* Lc705, *Bifidobacterium breve* 99, and *Propionibacterium freudenreichii* JS was ineffective in reducing AOM recurrence or nasal colonization by bacterial pathogens. Probiotic treatment, however, decreased recurrent upper respiratory tract infection (URTI) [32], suggesting probiotics' effectiveness against viral respiratory infections.

The primary objectives of this study were to examine by qPCR the prevalence and prolonged presence (persistence) of HBoV1-4 in the nasopharynx of otitis-prone children, and learn whether a probiotic combination might reduce HBoV prevalence/persistence during the cold season. In addition, we looked for associations between HBoV and each child's characteristics, respiratory symptoms, or AOM pathogens.

2. Materials and methods

2.1. Children

The study protocol was approved by the ethics committee of Helsinki University Central Hospital, with written informed consent from parents or guardians. Children were recruited from newspaper advertisements, primary health care centres, daycare centres, and on the internet. Children were classified as otitis-prone if they had ≥ 4 AOM episodes during the preceding 12 months or ≥ 3 episodes during the preceding 6 months. Those children who had undergone adenoidectomy or tympanostomy were accepted if they had suffered the required number of AOM episodes.

This research was conducted in conjunction with other substudies [6,33], with a study population part of a larger project described by Hatakka et al. [32]. Briefly, in a double-blind, placebo-controlled, randomized, 6-month intervention study between September 2001 and April 2002, originally 269 otitis-prone children (from 9 months to 5.6 years old) consumed daily either one capsule of probiotics (*L. rhamnosus* GG, *L. rhamnosus* Lc705, *B. breve* 99, and *P. freudenreichii* JS) (n = 135) or placebo (n = 134). NPS samples were collected as described [6,32,33] at the scheduled baseline visit in autumn, at the first follow-up visit after 3 months in winter, and at the final visit after 6 months in spring. For the present study, all three NPS samples were available from 152 otitis-prone children (105 in the placebo and 47 in the probiotic group). Parents received advice to avoid days when the child had respiratory symptoms when making scheduled collection visits. Parents had to keep daily diaries, including signs and symptoms of AOM and respiratory infections, such as fever, earache, otorrhoea, rhinitis, cough, sore throat, chest wheezes, or night restlessness, and listing visits to health care authorities, and of the use of any medication.

2.2. AOM pathogen detection

NPS from the baseline visit, and 3-month, and 6-month visits were analyzed by PCR followed by hybridization for rhinoviruses and enteroviruses, and from the baseline visit, and 6-month visit by inoculation on sheep blood- or chocolate-agar plates for *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, as described [6,32].

2.3. Quantitative PCR for HBoV detection

DNA was purified from 200 µl of sample with the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. Initially, real-time quantitative PCR specific for the HBoV nucleoprotein-1 gene was performed as described [15,27,34]. Further, the multiplex reactions for detection of HBoV2-4 were executed according to Kantola et al. [35]. The criteria for a positive reaction were a cycle threshold of 40 cycles and a fluorescence count of 10.5. Minimum genome viral load allowing reproducible quantification was 10 copies per reaction, corresponding to 500 copies/ml for original NPS specimens.

2.4. Statistical analysis

HBoV was assessed as positive or negative by four positivity criteria (>100, >1000, >10,000 and >100,000 copies/ml of sample). Cochran's Q-test and adjusted pairwise comparisons served to analyze the changes in HBoV DNA-positivity between scheduled visits. The Chi-squared test served to analyze associations between the categorical baseline characteristics of children and HBoV prevalence and persistence. We analyzed the association between HBoV DNA-positivity and respiratory symptoms from 2-week (sampling day ± 1 week) and 4-week (sampling day ± 2 weeks) time-periods with logistic regression analysis, and GEE (generalized estimating equations) using information on respiratory symptoms provided by parents. The presence of respiratory symptoms in HBoV DNA-positive children we compared to those of HBoV DNA-negative children, with results as odds ratios (OR) with 95% confidence intervals. In addition, logistic regression and GEE analyses allowed study of association between AOM pathogens and the HBoV DNA-positivity. Logistic regression analysis allowed study of any possible effect of probiotic intervention on HBoV. Results are unadjusted (crude) and baseline-adjusted odds ratios (OR) with 95% confidence intervals. GEE analysis allowed inclusion of 3- and 6-month visits simultaneously and baseline positivity was a categorical covariate.

p-Values <0.05 were considered statistically significant. Data were analyzed with SPSS version 18.0 (SPSS Inc. Chicago, IL, USA).

3. Results

3.1. Presence and persistence of HBoV DNA

Of 269 otitis-prone children, 152 children aged 9 months to 5.6 years (mean 2.2 years) were examined for HBoV1-4 nasopharyngeal carriage during the cold period from September 2001 to April 2002. Of these, 26 (17.1%) exhibited a high load (>10,000 copies/ml of sample) of HBoV DNA (Table 1). HBoV2-4 DNA was undetectable in any of the study children. HBoV DNA was detected at all three visits, with the highest occurrence at 3 months (Fig. 1A). At the initial visit, 3.3% of the children carried a high load of HBoV DNA. After 3 months, the HBoV DNA prevalence among the NPS samples increased to 10.5%, but after 6 months, decreased to 7.9%. The change in HBoV DNA prevalence was statistically significant (Cochran's Q test,

Table 1

Number of children (%) with their first occurrence of HBoV DNA in the NPS among 152 otitis-prone children, with viral loads ranging from 1 000 to 100 000 copies/ml of sample during the 6-month study period.

Viral load (copies/ml)	Baseline[a]	3 mo[b]	6 mo	Total
>1000	11 (7.2)	21 (13.8)	11 (7.2)	43 (28.3)
>10,000	5 (3.3)	14 (9.2)	7 (4.6)	26 (17.1)
>100,000	1 (0.7)	8 (5.3)	3 (2.0)	12 (7.9)

[a] Unknown whether first-time positives.
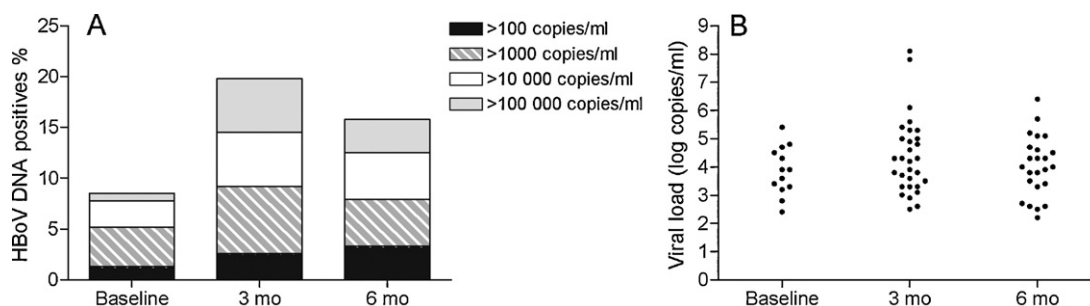[b] mo, months visits.

Fig. 1. (A) Distribution (%) of viral loads of HBoV DNA in the nasopharynx of 152 otitis-prone children within the 6-month study period. (B) Distribution of detectable HBoV viral loads (range 1.6×10^2 to 1.3×10^8 copies/ml) for all studied 456 nasopharyngeal samples. mo = months visits.

$p = 0.028$) due to the increase from baseline to 3 months (post hoc test, $p = 0.025$). The HBoV-DNA load ranged from 1.6×10^2 to 1.3×10^8 copies/ml of NPS sample (Fig. 1B).

Of 152 children, 16 (10.5%) showed the prolonged presence of HBoV DNA for at least 3 months, and one child for 6 months. In addition, 2 (1.3%) children had one negative sample between two HBoV DNA-positive samples. In 7 children with persistent HBoV DNA, viral loads remained above 10,000 copies/ml of sample, whereas in 3 with viral loads initially below 10,000 copies/ml, the viral load increased 10 to 100-fold.

3.2. Baseline and clinical characteristics

HBoV prevalence was significantly higher among children with ≥2 siblings than among those with 0–1 siblings (42.1% vs. 23.7%, $p = 0.029$). Moreover, children cared for at home showed less persistent HBoV DNA ($p = 0.075$) (Table 2). Other risk factors for AOM, including age, gender, parental smoking, use of a pacifier, and AOM episodes before or during the study, were not associated with HBoV prevalence or persistence – nor was number of days with respiratory symptoms.

Table 2
Distribution (%) of baseline characteristics of 152 children and their HBoV prevalence and persistence in the nasopharynx, by three positivity criteria, in groups of baseline characteristics. HBoV was regarded positive by first occurrence at baseline, at 3 months, or at 6 months visits, and the requirement for persistence was at least two consecutive positive (>100 copies/ml) samples. The Chi-squared test was used to test the associations between baseline characteristics and HBoV positivity. p-Values below 0.05 were considered significant.

Characteristic	Distribution (%)	HBoV-positive (copies/ml) (%)			
		>1000	>10,000	>100,000	Persistence
Age					
<3 years (n=125)	82.2	29.6	17.6	8.0	9.6
≥3 years (n=27)	17.8	22.2	14.8	7.4	14.8
Gender					
Girl (n=60)	39.5	31.7	20.0	8.3	10.0
Boy (n=92)	60.5	26.1	15.2	7.6	10.9
Siblings					
0 (n=52)	34.2	26.9	19.2	9.6	7.7
1 (n=62)	40.8	21.0	12.9	6.5	9.7
≥2 (n=38)	25.0	42.1[a]	21.1	7.9	15.8
Exclusive breast-feeding					
<3 mo (n=44)	28.9	31.8	20.5	6.8	6.8
≥3 mo (n=107)	70.4	27.1	15.9	8.4	12.1
Type of day care					
Home care (n=64)	42.1	32.8	18.8	6.3	6.3[b]
Small group care (n=21)	13.8	33.3	23.8	19.0	23.8
Large-group/day-care centre (n=67)	44.1	22.4	13.4	6.0	10.4
Use of pacifier					
No (n=38)	25.0	31.6	18.4.	10.5	10.5
Yes, previously (n=55)	36.2	29.1	16.4	7.3	12.7
Yes, at the moment (n=59)	38.8	25.4	16.9	6.8	8.5
Smoking in the household					
No (n=87)	57.2	24.1	14.9	10.3	13.8
Yes (n=65)	42.8	33.8	20.0	4.6	6.2
History of atopic diseases					
No (n=109)	71.7	27.5	18.3	8.3	10.1
Yes (n=43)	28.3	30.2	14.0	7.0	11.6
AOM episodes during the preceding 12 months					
<6 (n=76)	50.0	31.6	17.1	7.9	14.5
≥6 (n=74)	48.7	25.7	17.6	8.1	6.8
AOM episodes during the study					
≥1 (n=96)	63.2	28.1	17.7	5.2	N/A
≥3 (n=25)	16.4	36.0	20.0	8.0	N/A
Days with respiratory symptoms during the study					
<35 (n=50)	32.9	28.0	18.0	10.0	8.0
35–55 (n=51)	33.6	27.5	15.7	7.8	15.7
>55 (n=51)	33.6	29.4	17.6	5.9	7.8

[a] Chi-squared test: $p = 0.072$.
[b] Chi-squared test: $p = 0.075$.

Table 3
Presence of otitis pathogens in the samples, and their association with the presence of HBoV DNA by two different criteria of positivity. Generalized estimating equations (GEE) analysis with binary logistic regression analysis served to calculate the odds ratios OR (95% CI), when the pathogen-positive children are compared with the negative children.

AOM pathogen	No. of positive/all samples	HBoV (copies/ml)					
		>1000			>10,000		
		OR	95% CI	p	OR	95% CI	p
Streptococcus pneumoniae	143/304	0.88	0.41–1.88	0.741	0.77	0.29–2.04	0.598
Haemophilus influenzae	67/304	1.58	0.72–3.46	0.251	1.58	0.55–4.51	0.396
Moraxella catarrhalis	131/304	1.23	0.59–2.57	0.584	0.86	0.32–2.33	0.768
Rhinovirus	76/420	0.99	0.47–2.10	0.984	1.08	0.42–2.74	0.874
Enterovirus	93/420	0.92	0.47–1.84	0.823	0.58	0.28–1.19	0.135

Table 4
Number (%) of HBoV DNA-positive children based on different positivity criteria in the study groups. The requirement for persistence was at least two consecutive positive (>100 copies/ml) samples.

HBoV positivity (copies/ml)	Time	Probiotic (%) n = 47	Placebo (%) n = 105	Probiotic vs. placebo[a]					
				Crude			Baseline-adjusted[b]		
				OR	95% CI	p	OR	95% CI	p
>100	Baseline	5 (10.6)	8 (7.6)						
	3 mo/6 mo[c]	9 (19.1)	34 (32.4)	0.49	0.21–1.14	0.098	0.44	0.18–1.06	0.067
>1000	Baseline	4 (8.5)	7 (6.7)						
	3 mo/6 mo	8 (17.0)	30 (28.6)	0.51	0.21–1.22	0.133	0.47	0.19–1.15	0.098
>10,000	Baseline	2 (4.3)	3 (2.9)						
	3 mo/6 mo	3 (6.4)	20 (19.0)	0.29	0.08–1.03	0.055	0.25	0.07–0.94	0.039
>100,000	Baseline	0 (0.0)	1 (1.0)						
	3 mo/6 mo	2 (4.3)	9 (8.6)	0.47	0.10–2.28	0.352	0.42	0.08–2.11	0.294
Persistence		4 (8.5)	12 (11.4)	0.72	0.22–2.36	0.589			

[a] Logistic regression.
[b] Adjusted for baseline positivity (>100 copies/ml).
[c] mo, months visits.

3.3. AOM pathogens

Of the children, 67.8% had *S. pneumoniae*, 34.9% *H. influenzae*, and 62.5% *M. catarrhalis* detected at the baseline and at 6-month visits, and 45.7% rhinovirus, and 41.0% had enterovirus detected at all study visits. At the baseline visit the co-detection rates of HBoV DNA-positive samples was 12.5% for rhinovirus and 7.7% for enterovirus. At the 3-months visit, the co-detection rates were 25%, and 18.2%, and at the 6-months visit, the co-detection rates were 14.3%, and 19.0%, respectively. No association appeared between the presence of HBoV DNA and AOM pathogens (Table 3).

3.4. Respiratory symptoms

All children experienced respiratory symptoms (fever, earache, cough, rhinitis, sore throat, chest wheezing, or discharge from the ear) during their 6-month follow-up; median (IQR) number of days with respiratory symptoms was 46 (33–68). No association appeared between HBoV DNA-positive samples (>10,000 copies/ml) and respiratory symptoms, either 1 or 2 weeks before and after each sample collection. In addition, HBoV DNA positivity, although analyzed only in the placebo group, showed no association with individual respiratory symptoms such as cough or wheezing.

3.5. Probiotic intervention

Probiotic supplementation reduced significantly the number of HBoV DNA-positive samples (>10,000 copies/ml) during the intervention period (probiotic vs. placebo: 6.4% vs. 19.0%, baseline adjusted OR = 0.25, CI 95% = 0.07–0.94, p = 0.039). A similar, though not statistically significant, reduction occurred when the results were analyzed by GEE (baseline adjusted OR = 0.45, CI 95% = 0.12–1.66, p = 0.228) or when applying another HBoV-positivity criterion (Table 4).

In addition, to allow time for the intervention to take place, we included only the baseline HBoV-negative children and analyzed the HBoV-positive children (by first occurrence of HBoV), and found less HBoV in the probiotic group (probiotic vs. placebo: 6.7% vs. 17.6%, OR = 0.33, CI 95% = 0.09–1.20, p = 0.092). Probiotic intervention did not, however, reduce the occurrence of prolonged presence of HBoV over 3 months (OR 8.5% vs. 11.4%, OR = 0.72, CI 95% = 0.22–2.360, p = 0.589).

4. Discussion

Human bocavirus was commonly present in the nasopharynx of otitis-prone children during the cold period even when they were free of respiratory symptoms. Altogether HBoV DNA was detectable from 43 (28.3%) children, and from 56 (12.2%) samples with a viral load of >1000 copies/ml of original sample. No HBoV2-4 DNA from their NPS samples was detectable. However, HBoV2 rarely occurs in respiratory secretions,[11,13,35–37] and HBoV3 and HBoV4 have been identified only in stool samples [11,35,37,38].

HBoV typically occurs in respiratory samples of children under age 3, when hospitalized for lower respiratory tract disease [19,39], whereas in adults, HBoV is rare [17,40–42]. However, recent studies report HBoV DNA in the upper airways also of asymptomatic children [43,44]. Of our 152 children who should have visited the scheduled sampling when they were asymptomatic, HBoV DNA was detectable in the NPS of 28.3%, of whom 17.1% showed a high viral load. This finding could be explained by the fact that these

children, otitis-prone, were thus more susceptible to viral infections.

Studies of consecutive respiratory samples show that HBoV DNA can persist/recur in the nasopharynx for several months [44–46]. We observed a prolonged presence of HBoV DNA of at least 3 months in 16 children, 72.2% of whom had a high viral load. In one child, HBoV DNA persisted throughout the entire 6-month study period. In otitis-prone children, any increased mucus or abnormal nasopharyngeal structure may favor HBoV persistence. In addition, we identified two children, with HBoV-positive NPS samples taken 6 months apart with a negative sample between. This finding could be attributed to mucosal contamination, differences in mucus secretion, fluctuating virus shedding, or re-infection. It is also likely that HBoV was occurring in the nasopharynx asymptomatic between visits, and may influence the detection of persistence.

Quantitative PCR detection of HBoV offer insight into the clinical impact of HBoV, because HBoV at a high viral load (>10,000 copies/ml) has been associated with RTIs. Below this cutoff, however, HBoV appears often as an innocent bystander or a remnant of past infection [15,27,34]. Thus, HBoV PCR positivity in the nasopharynx alone is insufficient to implicate the HBoV pathogenesis in acute RTI [20,27,34,47,48]. With only HBoV PCR positivity in NPS as the criterion for diagnosis, some cases of acute HBoV infection, evidenced by viremia, serodiagnosis, or both would be missed. Moreover, false diagnoses may ensue, perhaps as a result of persistent HBoV DNA after the primary infection, or of mucosal contamination. Serological testing, coupled with qPCR, could aid in study of trustworthy causal associations between HBoV and respiratory disease [27,34,48] In line with this, we confirmed that presence of HBoV DNA in NPS, even at high viral loads, did not associate significantly with respiratory symptoms reported 2 weeks before or after any scheduled sample-collection periods. Children should have visited the centres for sampling when they were healthy; no major respiratory symptoms were thus expected at sampling times. Regarding associations between baseline characteristics and prevalence of HBoV in children during 6 months, number of siblings and type of daycare may have been associated with HBoV prevalence or persistence. Higher number of siblings correlated with a high HBoV prevalence, perhaps because susceptibility to HBoV infection and mucosal contamination may fall when fewer siblings spread the virus.

Viral infections in the nasopharynx may prime a superinfection by bacterial pathogens through some unknown mechanism. For instance S. pneumoniae plays a major role in development of pneumonia associated with influenza-, parainfluenza-, and respiratory syncytial viruses [49]. Here, HBoV DNA in the nasopharynx was not associated with the presence of bacterial pathogen (S. pneumonia, H. influenzae, M. catarrhalis), or with rhino- or enteroviruses. Interestingly, in children with AOM, Beder et al. found a positive correlation between HBoV and S. pneumoniae in middle-ear fluids (MEF) [25]. Because we had no MEF samples available, we cannot confirm this.

Antibiotics are common for treatment against AOM, although AOM is usually self-limited, and respiratory viruses are also involved in the clinical course. Recurrent use of antibiotics leads to antibiotic resistance, and disturbances in microflora balance, and facilitates further colonization by AOM pathogens [28,29]. Probiotics could serve as alternative therapy to prevent infectious diseases as they balance the microbial equilibrium, enhance mucosal immunity, and compete with other bacterial adhesion sites [50]. In otitis-prone children, combination of probiotics (L. rhamnosus GG, L. rhamnosus Lc705, B. breve 99, and P. freudenreichii JS) failed to reduce AOM recurrence or nasal colonization of bacterial pathogens [32]. Nevertheless, probiotic treatment reduced recurrent URTI, which may imply probiotics' effectiveness against respiratory infections of viral etiology. In particular, the probiotic strain L. rhamnosus GG has been effective in prevention of children's upper respiratory tract infections [30,31]. Interestingly, we found that probiotic treatment reduced significantly the number of HBoV DNA-positive samples ($p = 0.039$) in 3–6 months after intervention, and marginally also the amount of HBoV-positive children. However, as the number of children in our study groups was unequal, further studies are necessary in order to confirm this observation.

5. Conclusions

In conclusion, HBoV commonly occurs in the upper airways of otitis-prone children, although in the nasopharynx it showed no correlation with respiratory symptoms. In addition, prolonged or recurrent HBoV DNA may be observable for 3–6 months. Probiotic treatment is possibly effective against HBoV.

Conflict of interest

Katja Hatakka is an employee of Valio Ltd. Other authors disclose no conflicts of interest.

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