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# 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.



## 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 bocaviruses (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 gastroenteritis [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 $\geq$4 AOM episodes during the preceding 12 months or $\geq$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.