Original Article

Role of Human Bocavirus in Upper Respiratory Tract Infections and Acute Otitis Media

Johanna Nokso-Koivisto,[1,a] Richard B. Pyles,[1,2] Aaron L. Miller,[1] Kristofer Jennings,[3] Michael Loeffelholz,[4] and Tasnee Chonmaitree[1,4]

Departments of [1]Pediatrics, [2]Microbiology and Immunology, [3]Preventive Medicine and Community Health, and [4]Pathology, University of Texas Medical Branch, Galveston

Corresponding Author: Tasnee Chonmaitree MD, University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555-0371. E-mail: tchonmai@utmb.edu.

[a]**Present Affiliation:** Department of Otorhinolaryngology, Helsinki University Central Hospital, PO Box 220, 00290 Helsinki, Finland.

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Background. Human bocavirus (HBoV) is a newly described parvovirus. HBoV1 has been associated with respiratory infections, including acute otitis media (AOM), but the knowledge on the significance of HBoV1 in upper respiratory tract infections (URI) and AOM in relation to other respiratory viruses is limited. The objective of this study was to compare the rate of detection of HBoV1 to that of other respiratory viruses in specimens from children with URI, with and without AOM complication.

Methods. Nasopharyngeal secretions (NPS) were collected during URI from healthy children (6–35 months) followed prospectively for 1 year; specimens have been previously analyzed for broad spectrum of respiratory viruses. Archived NPS were analyzed for HBoV1 using a high-throughput, quantitative polymerase chain reaction method.

Results. Seven hundred and seven NPS samples collected during URI episodes from 201 children were studied for HBoV1. A total of 94 (47%) children tested positive for HBoV1 DNA during 172 (24%) URI episodes; HBoV1 was present as the only virus in 44 (6%) URI episodes. Overall, 37% of URI episodes were complicated by AOM. Of URI associated with single virus (n = 303), the rate of AOM complicating URI associated with HBoV1 only was 52% (23 of 44); this was a higher AOM rate, compared to that of other respiratory viruses.

Conclusions. Among URI associated with single respiratory virus, HBoV1-URI was commonly associated with AOM complication. The important role of HBoV1 on AOM pathogenesis needs to be studied further.

Key words. acute otitis media; children; human bocavirus; upper respiratory tract infection.

BACKGROUND

Human bocavirus (HBoV) is a parvovirus that was first discovered in 2005 [1]. To date, 4 different HBoV species, HBoV 1–4, have been reported and associated with different clinical manifestations [1–7]. The HBoV1 is primarily a respiratory virus; the other species [2–4] are more commonly related to gastrointestinal tract [7].

Acute otitis media (AOM) is one of the most common infections in children. Acute otitis media usually occurs concurrently with or just after viral upper respiratory tract infection (URI), and certain respiratory viruses, eg, respiratory syncytial virus (RSV), adenovirus, and rhinovirus have commonly been associated with AOM [8–10]. Beder et al. [11] detected HBoV in 6.3% of nasopharyngeal secretions (NPS) from children with AOM, and HBoV-DNA was also found in 2.7% of middle ear fluids (MEFs). In AOM with otorrhea, 4% of MEF samples were positive for HBoV [12]. Two studies with hospitalized patients reported that 33%–44% of children with respiratory infection and HBoV detection had AOM [4, 13]. In a recent study, HBoV1 infection, confirmed by serology, was associated with AOM in children [14].

Human bocavirus-DNA has been detected in symptomatic and asymptomatic children [13, 15] and repeatedly from

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the same subjects [15]. The cause-and-effect relationship of HBoV detection in respiratory specimens and the presence of HBoV in children over time still need to be studied further. We investigated the presence of HBoV1-DNA in NPS from young children with URI to determine the role of HBoV1 in URI and AOM, and we compared the rate of AOM complicating HBoV1-URI to that of URI associated with other viruses.

STUDY DESIGN

Study Design and Subjects

Specimens tested for HBoV1 were archived specimens from a prospective, longitudinal study performed between January 2003 and March 2007 at the University of Texas Medical Branch (Galveston, TX) [10]. Constitutionally healthy children were enrolled at the ages of 6–35 months and followed for 1 year for occurrences of URI and AOM. The parents informed the study personnel when the child developed URI symptoms. Children were seen by a study physician and followed after URI onset for the occurrence of AOM. At each visit, otoscopic and physical examinations and tympanometry were performed. Acute otitis media was considered to have complicated URI if it occurred within 28 days of URI onset. Acute otitis media was defined as acute onset of symptoms, signs of tympanic membrane inflammation (erythema, opacification, or bugling), and the presence of fluid in the middle ear. The study was approved by the Institutional Review Board of University of Texas Medical Branch, and informed consent was obtained from the guardians of all study children.

Virologic Studies

Respiratory specimens for viral studies were collected at the initial URI visit and when AOM was diagnosed. Nasal swabs were collected for viral culture and NPS for other viral studies. The NPS sample was collected by vacuum suction catheter with mucus trap, which were rinsed with 1 mL phosphate-buffered saline. The total secretion volume was recorded to provide the dilution factor of the original sample.

Respiratory specimens were collected during 864 URI episodes in the original study [10]. The NPS samples collected during RSV season were also analyzed for RSV antigen detection by enzyme immunoassay (EIA). Culture and RSV-EIA-negative samples were tested by real-time polymerase chain reaction (PCR) for adenovirus, enterovirus, rhinovirus, and coronavirus (OC43, 229E, and NL63) and by microarray PCR for RSV A and B, parainfluenza viruses 1–3, and influenza viruses A and B, performed at the Medical College of Wisconsin (Milwaukee, WI). The HBoV1 and human metapneumovirus (hMPV) were not targeted in the assay performed and reported in the original study. Specific to this report, 707 frozen archived NPS specimens were available for testing by quantitative PCR (qPCR) for hMPV, HBoV, and RSV; these represent 81% of the URI samples collected for viral studies. All of the results from previous and new virological analysis of these 707 samples were included in this study.

Nucleic acid extraction and qPCR were performed in a "clean room" facility within the Galveston National Laboratory (University of Texas Medical Branch) using MagMAX Total Nucleic Acid isolation kits (Ambion/Applied Biosystems, Austin, TX) and a Biosprint 96 (QIAGEN, Valencia, CA). A customized script directed the extraction protocol to optimally recover RNA and DNA from each sample. After extraction, the elution volume (200 µL) was diluted 1:1 with nuclease-free 0.1 mM EDTA (Ambion/Applied Biosystems, Austin, TX) and distributed to daughter plates for subsequent analyses.

DNA from each sample was evaluated using a duplex qPCR assay with primers that amplified targets within the HBoV1 NS-1 region [16] and human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) [17]. Glyceraldehyde 3-phosphate dehydrogenase was used as an indicator of sample integrity and extracted nucleic acid quality. TaqMan probes were used to track the specific amplification in the duplex. Quantitative PCR was completed in a C1000 thermocycler equipped with a CFX reaction module (Bio-Rad) using the following parameters: Cycle 1: 95°C (3 minutes); Cycle 2, Step 1: 95°C (15 seconds), Step 2: 60°C (45 seconds), repeated 50 times. Fluorescent signal data were collected at the end of each annealing and extension step. Viral genomic titers were extrapolated from standard curves of plasmids harbouring the PCR targets generated in parallel for each run. A parallel qPCR reaction for GAPDH [17] was completed on every clinical sample to evaluate RNA and cDNA quality. Detection of hGAPDH at less than 500 copies/reaction from the NPS sample suggested inadequacy of the specimen and led to the exclusion of the sample from further analysis. Viral load was calculated as HBoV1-DNA copies/mL of the original specimen based on the original dilution of the specimens and subsequent assay dilutions. Due to unknown dilution of 58 NPS samples, viral load analysis was available in NPSs from 649 URI episodes.

In addition to HBoV1 qPCR, duplex hMPV-RSV-qPCR was also performed; hMPV data have been reported previously [18]. Virology results reported here combined data from qPCR and data from the original study [10]. For RSV, previous PCR data were performed only in culture-negative and EIA-negative samples by microarray, which is less sensitive than real-time PCR [19]. As a result, new

detection of RSV was made by qPCR in 71 URI episodes, including 38 episodes with RSV as the single virus.

Statistics

Comparisons were made using Fisher's exact test for 2 × 2 tables and *t* tests. For viral load, log transformations were applied to make standard deviations comparable. To compare number of URI episodes, Poisson regression with a categorical predictor was used and assessed for goodness-of-fit. All calculations were carried out in R, a software environment for statistical computing and graphics (http://cran.r-project.org).

RESULTS

The HBoV1 testing was performed in 707 NPS samples collected during URI from 201 children. Demographic characteristics and risk factor data are presented in Table 1, which also compares data from children who had 1 or more URI episode with HBoV1 detection with those without. Children with HBoV1 infection had significantly more frequent URI episodes, compared with children without HBoV1 infection; they also have more URI episodes associated with multiple viruses. Of 94 children with HBoV1 infection, HBoV1 was detected more than once in 42 (45%) children during the year. In 23 children, HBoV1 was detected twice: in 10 children, 3 times; in 5 children, 4 times; in 2 children, 5 times; in 1 child, 6 times; and 1 child had 8 HBoV-positive URI episodes during the study period. The pattern of multiple HBoV1 detections varied in the 42 children: multiple detection in consecutive samples, which suggests persistence (n = 23); 2 or more HBoV1 detections with at least 1 negative detection in between, which suggests either reinfection or periodic shedding (n = 19). The interval between HBoV1-positive URI episodes ranged from 8 to 301 days (with or without HBoV1-negative episodes in between).

Respiratory viruses were detected in 542 (77%) NPS samples; of these, 303 (43%) contained a single virus. HBoV1 was detected in 172 (24%) URI episodes (Table 2). In 44 URI episodes (6% of all episodes), HBoV1 was the only respiratory virus detected; in 128, another virus was also detected, most often adenovirus, enterovirus, and rhinovirus (46, 40, and 36 episodes, respectively). The HBoV1 was detected year-round, with 63% between October and March. There was no significant difference in signs and symptoms at the time of sick visit between the children with HBoV1 as a single virus and the children without HBoV1 (data not shown).

Overall, 37% of all URI episodes, 39% of virus-positive URI episodes (single or multiple viruses), and 45% of HBoV1-positive episodes (single or multiple viruses) were complicated by AOM. Of URI associated with the presence of a single virus, the rate of AOM complicating URI was 52% in HBoV1-positive episodes. The rates of AOM complicating URI for other viruses are presented in Table 2. Although presence of HBoV1 alone in the child with acute onset of URI, without other viruses detected, suggests the association between HBoV1 and acute URI

Table 1. Demographic and Individual Characteristics of 201 Study Children

	Children With HBoV1 Infection[a] (patients, n = 94)	%	Children Negative for HBoV1[a] (patients, n = 107)	%
Female	45	48	54	51
Median age at enrollment (mo)	12 (range, 6–34)		12 (range, 6–35)	
Number of URI episodes/child year (median)[b]	4.8		2.4	
Number of URI episodes with ≥2 viruses/child year (median)[b]	2.1		0.4	
URI episodes with ≥2 viruses/all URI episodes[b]	197 of 448	44	42 of 259	16
Race				
Asian	3	3	3	3
Black	28	30	29	27
Biracial	8	9	11	10
White	55	59	64	60
Ethnicity: Hispanic/Latino	37	39	52	49
Childcare arrangement				
Home	60	64	72	67
Home day care	7	7	9	8
Day care center	26	28	26	24
Breast feeding[c]	50	53	55	51
Cigarette smoke exposure	34	36	29	27
History of prior otitis media episodes[b]	65	69	48	45

Abbreviations: HBoV1, human bocavirus 1; URI, upper respiratory tract infection.

[a]One or more HBoV1-positive result(s) during 1-year study period.

[b]Significant difference between the groups, Fisher's exact test for 2 × 2 tables, $P < .001$.

[c]Any breast feeding irrespective of the duration.

Table 2. Respiratory Viruses Detected During 707 Upper Respiratory Tract Infection Episodes

Virus	URI Episodes, n (%[a])	Episodes With Single Virus, n	Percentage of AOM Related to Single Virus
HBoV1	172 (24)	44	52%
Adenovirus	164 (23)	50	48%
Rhinovirus	145 (21)	59	25%
RSV	105 (15)	41	44%
Enterovirus	103 (15)	31	32%
Coronavirus	56 (8)	11	45%
hMPV	48 (7)	25	24%
Parainfluenzavirus	43 (6)	26	27%
Influenzavirus	31 (4)	16	31%
			Percentage of AOM Overall
Single virus	303 (43)		37%
Combined viruses	239 (34)		41%
Virus-negative	165 (23)		33%
Total	707[b]		37%

Abbreviations: AOM, acute otitis media; HBoV, human bocavirus; hMPV, human metapneumovirus; RSV, respiratory syncytial virus; URI, upper respiratory tract infection.

[a] Percentage of total number of URI episodes.

[b] Total number of viruses = 867.

symptoms, persistence of HBoV1 from previous episodes could not be ruled out. We used available longitudinal data to determine whether these children had shed HBoV1 previously. In 44 URI episodes (35 children), 15 episodes were associated with previous shedding of HBoV1 10–89 days previously. Of the remaining 29 episodes, there was no HBoV1 detected in the previous URI episode in 15; previous data were not available in 14 episodes (first URI episode in the study). The rate of AOM after URI was 52% (15 of 29) in cases without documented previous HBoV1 detection and 40% (6 of 13) in cases in which available data excluded HBoV1 persistence.

Viral load determination was available in samples collected during 157 of 172 (91%) episodes that were positive for HBoV1. The median HBoV1 viral load of all positive samples was 2.7×10^6 copies/mL of the original volume; the mean viral load was 2.7×10^{10} copies/mL (range, 3.2×10^3 to 1.7×10^{12} copies/mL). The HBoV1 viral load did not correlate with any URI sign or symptom or development of AOM (data not shown).

DISCUSSION

In this longitudinal study to determine URI and AOM occurrences in young children, we frequently detected HBoV1 alone or in combination with other viruses during URI. The unique feature of our study is the comparison of the rate of AOM complicating URI associated with single respiratory virus. We found that a high proportion of HBoV1-associated URI episodes were complicated by AOM. Our findings suggest that HBoV1 may play an important role in AOM pathogenesis, similar to other viruses such as RSV, adenoviruses, and coronaviruses [10, 20].

The HBoV1 is now recognized as a respiratory virus [5]; it has been detected in the MEF of children with AOM [12] and active HBoV1 infection documented by serology significantly associate with AOM development [14]. However, it is known that HBoV-DNA can be detected in asymptomatic children [15] due to prolonged presence of the viral nucleic acids. Therefore, detection of HBoV1-DNA alone in the NPS might not be sufficient to document active HBoV1 infection. Unfortunately, we were unable to confirm active HBoV1 infection in our study by serology. Due to the longitudinal nature of the study and nonsevere nature of viral URI and AOM, we were not able to include blood drawing for acute and convalescent serology in our design. Nevertheless, indirect evidence exists to support the role of active HBoV1 infection in our cases with URI associated with HBoV1 alone. First, we used extensive viral diagnostic methods to detect a variety of common respiratory viruses during symptomatic URI, although we did not detect less common, newly described viruses such as polyomaviruses and parechoviruses. Negative findings for other viruses but positive finding for HBoV1 only suggested the association between HBoV1 (a recognized respiratory virus) and acute symptoms. Second, viral load in the cases of HBoV1 alone was relatively high (mean viral load, 1.8×10^{10} copies/mL of the original sample; range, 3.2×10^3 to 5.5×10^{11}). Lastly, in our recently published study using NPS samples from the same cohort [21], concentrations of lactate dehydrogenase, a marker for cellular injury during inflammation, are associated with the rate of AOM complication after URI and with the presence of adenovirus, rhinovirus, and HBoV1. These findings together support the association between the presence of HBoV1 and its clinical significance during URI episodes for which no other virus was detected.

The incidence of HBoV1 detection in our patients with URI (24%) is among the highest published; overall, the incidence of HBoV has ranged from 5% to 33% in children with respiratory infection [2–4, 15, 22–23]. The higher incidence of HBoV detection in longitudinal studies, compared with that in cross-sectional studies, is likely from prolonged presence of the virus in the respiratory tract. The same reason may also explain high frequencies of concurrent detection of HBoV with other viruses, which has been reported to occur up to 72% [13, 15, 22, 24–25]. In our study, 45% of children with HBoV1 in the NPS had the virus detected more than once. Prolonged shedding of HBoV1 in nasal secretions has been described up to 11 weeks in children attending day care [15] and up to 3 months in otitis-prone children [26]. Extended viral shedding has also been observed with other DNA-viruses; in the same study population, we studied genetic sequences of adenoviruses and found that repeated presence could be from the same viral serotype and strain detected continuously or intermittently, or different serotype or strain detected sequentially [27]. Further studies of the viral genome in children with sequential HBoV1 are required, to differentiate between prolonged presence of the viral and reinfection with different HBoV isolates. These types of studies will help elucidate the natural history and foster the understanding of the pathogenicity of HBoV1 infection.

In conclusion, HBoV1 was a common virus detected alone or in combination with other respiratory viruses during URI. Among cases of URI associated with presence of single virus, HBoV1-URI was associated with a high rate of AOM complication.

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Potential conflict of interest. All authors: No reported conflicts.

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