TITLE PAGE

Brief Report

Title: No Correlation Between Nasopharyngeal Human Bocavirus 1 Genome Load and mRNA Detection or Serology in Adeno-/tonsillectomy Patients

Running title: HBoV1 in Nasopharynx and Tonsils

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Summary

Relatively high loads of HBoV1 DNA can be detected in the nasopharynx of asymptomatic subjects, which are negative for mRNA and/or serodiagnostic markers. HBoV1 DNA quantitative PCR may have lower specificity than HBoV1 mRNA detection for diagnosing symptomatic infection.

FOOTNOTE PAGE

Conflict of interest

Dr. Allander has a patent Human bocavirus and methods of diagnosis and treatment licensed to Karolinska Institutet Innovations AB. Other authors report no potential conflicts of interest, including relevant financial interests, activities, relationships and affiliations.

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Meetings

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ABSTRACT

Human bocavirus 1 (HBoV1) can persist in nasopharynx and tonsils. Using HBoV1 serology, reverse-transcription polymerase chain reaction (PCR) for detecting messenger RNA (mRNA) and quantitative PCR for HBoV1 genome load count, we studied in what extent the HBoV1 DNA loads in nasopharynx correlates with acute infection markers. Tonsillar tissue, nasopharyngeal aspirate and serum were obtained from 188 elective adeno-/tonsillectomy patients. Relatively high loads of HBoV1 DNA were detected in the nasopharynx of 14 (7%) primarily asymptomatic subjects with negative mRNA and/or serodiagnostic results. Quantitative HBoV1 DNA PCR may have lower specificity than HBoV1 mRNA detection for diagnosing symptomatic infection.

Key words: bocavirus, parvovirus, nasopharynx, tonsil, serology, diagnosis, detection

BACKGROUND

Human bocavirus (HBoV) was discovered in 2005 and belongs to the *Parvoviridae* family [1]. It is a non-enveloped single-stranded DNA virus causing mild to life-threatening respiratory tract infections in young children. HBoV1 is primarily transmitted by the respiratory route [1]. Three other human bocaviruses (HBoV2-4) have been discovered in stool and are considered enteric. HBoV1 is a frequent finding in young children suffering from lower respiratory tract illnesses such as bronchiolitis, wheezing, asthma and pneumonia [1,2]. The persistence of HBoV DNA in the airways and tonsils has been under investigation lately. In a recent study, HBoV DNA was found in tonsil squamous cell carcinoma tumors, prompting speculations of a possible causal association [3,4]. The virus is known to persist for weeks or months in the respiratory tract whereby a qualitative polymerase chain reaction (PCR) is insufficient as a diagnostic tool [1,2,5,6]. Microbiological diagnosis is often incorrectly based on a qualitative multiplex PCR. The most reliable diagnosis of acute HBoV1 infection is considered to be based on messenger RNA (mRNA) or a high HBoV1 DNA load in nasopharyngeal aspirate (NPA), DNA in serum, and serology [1,2,5,7]. HBoV1 DNA has been shown to also persist in adenoids and tonsils of children [8]. The aim of this study was to evaluate if high HBoV1 DNA loads occur in NPA or tonsils in adeno-/tonsillectomy patients without acute HBoV1 infection, based on a documented lack of HBoV1 mRNA and/or IgM, the gold standards for diagnosis. We hypothesized that there is no active bocavirus replication in persistent HBoV1 detection.

METHODS

Study Population

Tonsil and nasopharyngeal samples were collected from 200 consecutive patients who underwent adeno-/tonsillectomy at the Satakunta Central Hospital, Pori, Finland, between April 2008 and March 2009. The inclusion criteria were tonsillectomy, adenotonsillectomy or adenotomy due to

clinical indication and written informed approval from the study subject or his/her parents. Out of the 200 enrolled patients, 12 yielded low-quality samples. In total, 188 patients with a median age of 12 years (range 1-65) underwent elective adeno-/tonsillectomy (n=143) or sole adenotomy (n=45) and had sufficient and good quality biopsy samples for microbial and immunological studies [9]. The main indications for tonsillectomy were recurrent tonsillitis in 43 (30%) and tonsillar hypertrophy in 48 (34%) of 143 patients and for adenotonsillectomy, adenotonsillar hypertrophy in 40 of 143 (28 %) patients, respectively [9]. Other indications (8%) for adeno-/tonsillectomy were e.g. throat abscess, recurrent fever, food remnants in tonsils and teeth braces. Indications for adenotomy were hypertrophy in 17 (38%) and recurrent otitis in 28 (62%) of 45 patients. All the study patients filled a standardized health questionnaire including respiratory symptoms 30 days before and after the operation [9]. On the operation day 127 (67%) had no respiratory tract symptoms, 37 (20%) reported mild respiratory symptoms and 24 (13%) had no data.

Samples

Adeno-/tonsillectomy was performed by otorhinolaryngologists according to routine clinical procedure. A part of the internal tonsillar tissue was instantly cut in 3-4 mm cubes, stored in RNAlater, an RNA stabilization reagent (Qiagen, Hilden, Germany), incubated at +2-8 ºC until the next working day and finally stored at -80 ºC [9]. Nasopharyngeal aspirate samples were collected using a standardized procedure. If the aspirate yield was small, the collection was repeated after administration of 2 ml physiologic saline. For viral analyses, a part of the tonsils and a nasopharyngeal aspirate were stored in dry tubes at -80 °C [9]. The first sample of the paired serum samples was collected during the tonsillectomy anesthesia and the follow-up sample was taken in a median of 58 days (range 36-104).

Ethical Approval

137 The study protocols were approved by the Ethics Committee of the Satakunta Central Hospital and
138 by the Ethics Committee of the Hospital District of Southwest Finland.
139
140 Virus Diagnostics
141 Virus diagnostics of all NPA and tonsil samples was performed according to clinical routine using
142 PCR. Adenoid tissue samples were not analyzed. In-house real-time PCR assays were used to detect
143 HBoV1, rhinovirus, enterovirus, and respiratory syncytial virus as described previously [9]. Seeplex
144 RV12 ACE Detection (Seegene, Seoul, Korea) multiplex PCR assay was used for detection of
145 adenovirus, coronaviruses (229E/NL63 and OC43/HKU1), influenza A and B viruses,
146 metapneumovirus, parainfluenza virus types 1-3, respiratory syncytial virus group A and B, and
147 rhinovirus according to manufacturer's instructions. Quantitative PCR (qPCR) was used for
148 measuring the HBoV1 DNA load [10]. Serological tests for HBoV1-specific IgM and IgG were
149 performed for 122 patients [5,11]. Serology of the adenotomy patients (n=45) was not analyzed. To
150 verify that the IgG results were HBoV1 specific, the serum samples were blocked with HBoV2 and
151 HBoV3 antigens. The mRNA expression levels of HBoV1 in NPA and tonsil samples were
152 analyzed by reverse-transcription PCR (RT-PCR) [7]. An RT-PCR detecting human beta-actin
153 mRNA was used as control for intactness of mRNA in the samples [12]. Virus PCR and qPCR were
154 done at the Department of Virology, University of Turku, Turku, Finland, and at the Department of
155 Clinical Microbiology, Karolinska University Hospital, Stockholm, Sweden. Serology was
156 analyzed at the Department of Virology, University of Helsinki and the RT-PCR at the Norwegian
157 University of Science and Technology, Trondheim, Norway.
158
159 RESULTS
160 HBoV1 DNA in NPA, tonsillar tissue, or in both samples, could be detected in 40 patients (21%)
161 with a median age of 5 years (range 1-22). These patients did not have severe respiratory tract

infection but 12 of 40 patients (30%) reported one or more of the following: mild rhinitis, cough, symptoms of otitis, throat pain or upper airway obstruction symptoms on the operation day. In the sole adenotomy group 8 of 15 patients (53%) and in the adeno-/tonsillectomy group 4 of 25 patients (16%), respectively, reported symptoms (Tables 1-2).

Twenty-eight patients were positive for HBoV1 DNA in NPA only, 7 in tonsillar tissue only and 5 in both samples. Five sole adenotomy patients had high (>10^6 copies/ml) viral load in NPA using qPCR but were mRNA negative (Table 1). In the tonsillectomy group 9 patients had relatively high (>10^4 copies/ml) viral load in NPA but were mRNA negative and corresponding sera available were HBoV1 IgM-negative (Table 2). Only 1 patient gave a (barely) IgM-positive test result, but with a stable IgG absorbance in paired samples (Table 2). In all but three patients, the HBoV1 DNA finding was accompanied with IgG positivity indicating a prior infection. These three HBoV1 DNA-positive but seronegative children had, however, prior HBoV2 immunity, which suggest that their HBoV1 IgG-negativity can be explained by an immunological phenomenon called original antigenic sin [13]. Furthermore, HBoV1-IgG levels did not increase in any of the 7 paired serum samples of HBoV1 DNA-positive patients (Table 2). All 29 NPAs and 8 tonsils analyzed were HBoV1-mRNA negative (Tables 1-2). Eight NPA samples with HBoV1 DNA loads >10^4 were tested with the beta actin-mRNA PCR, all with strongly positive results.

DISCUSSION

Our study confirms that HBoV1 can be found in the respiratory tract of patients with chronic and recurrent adenotonsillar disease. Quite a high prevalence (21%) of HBoV1 DNA in tonsils and/or NPA of elective adeno-/tonsillectomy patients was detected which agrees with earlier studies [8]. An even higher prevalence (43%) has been discovered in mainly asymptomatic subjects but the patients were small children (median age of 23 months) undergoing elective adeno-/tonsillectomy

187 and/or myringotomy [14]. We also found relatively high (>10^4 copies/ml) or high (>10^6 copies/ml)
188 HBoV1 DNA loads in nasopharynx of 13% and 3% our study patients, respectively. However, the
189 high DNA loads were not accompanied by positive HBoV1 mRNA or serological responses. Our
190 results supported the study hypothesis that HBoV1 was not actively transcribing in persistent
191 infection.
192
193 The most common laboratory diagnostic method for respiratory infections is qualitative PCR,
194 despite the fact that HBoV1 DNA can, due to prolonged presence or intermittent shedding, be
195 detected in the nasopharynx for months after a symptomatic respiratory infection [1,6,15]. Previous
196 studies have suggested that the DNA amount decreases over time and that high DNA loads (>10^4 to
197 10^6 copies/ml, depending on the study) would be a sign of acute bocavirus infection [2,5,7,15]. To
198 define one specific threshold for high viral load is very demanding due to the various test methods,
199 the type and quality of the specimens, and the time of collection. In our study we found high loads
200 (>10^6 copies/ml) of HBoV1 DNA particularly in adenotomy patients of which 3 were asymptomatic
201 and 2 had mild respiratory tract symptoms. Only 1 adeno-/tonsillectomy patient with relatively high
202 viral load (>10^4 copies/ml) reported symptoms.
203
204 In addition, mRNA of HBoV1 has been used as a marker of viral activity: HBoV1 mRNA can be
205 detected in NPA of patients with symptomatic respiratory tract infection but not in asymptomatic
206 controls [2,7]. It is known that HBoV1 DNA is stored in adenotonsillar tissue [8]. We wanted to
207 investigate the viral activity in tonsils. None of the tonsils showed HBoV1 mRNA regardless of the
208 HBoV-DNA load. Furthermore, all NPAs were also mRNA negative, in line with earlier studies of
209 non-acute HBoV1 infections[7,8]. Conversely, in previous studies, the detection of HBoV1 mRNA
210 in symptomatic patients was associated with high HBoV1 DNA loads [2,7]. In our elective adeno-
211 /tonsillectomy patients, relatively high loads of HBoV1 DNA in the respiratory tract were not

212 associated with concomitant viral replication demonstrated by the lack of mRNA detection. Our
213 data suggests that HBoV1 DNA or its high load by qPCR are less specific markers for acute
214 HBoV1 infection than mRNA, at least in adenotonsillar surgery subjects. In this respect, our data
215 support using HBoV1 mRNA detection as a more reliable method for diagnosing acute infection as
216 suggested previously [2,7,8].
217
218 Serological results were in line with the clinical findings and did not support acute HBoV1
219 infection in any patients. Since most patients studied by serology were ≥5 years of age, they most
220 likely have already experienced primary bocavirus infection. The HBoV1 DNA finding in the
221 respiratory tract was accompanied by IgG positivity in 18/25 cases (no sera available n=4), of
222 which 17 were IgM negative, indicating past infections. The one barely IgM-positive patient with
223 HBoV1 DNA in tonsils, showed an already high and stable IgG in paired samples, indicating a
224 recent but non-acute infection. In two earlier studies among wheezing children, there has been an
225 association of high ($>10^4$ or $>10^6$ copies/ml) HBoV1 DNA load with diagnostic serology [2,5].
226 This association could not be found in the current study of primarily asymptomatic tonsillectomy
227 patients due to lack of acute infections. We show that persisting HBoV1 DNA can be of relatively
228 high loads also in non-acute infections.
229
230 This study provides new information about HBoV1 DNA positivity without clinical
231 illness/manifestation and also confirms earlier results of HBoV1 diagnosis [2,5–7,15]. Earlier
232 studies have focused on young children with respiratory tract infection [5,7,14,15] whereas our
233 study had slightly older and mainly asymptomatic adeno- /tonsillectomy patients. A major
234 limitation of the current study is that the data set was not complete: 8 of the 45 (18%) HBoV1 PCR-
235 positive NPA or tonsillar tissue samples were not analyzed by mRNA RT-PCR. Another limitation
236 of this study is the low number of paired serum samples. Serum samples were not available at the

enrollment (n=4), at the follow-up visit (n=10) or both samples (n=4). Serology of the adenotomy group was not analyzed. However, this is still the largest study on subjects without acute respiratory symptoms that compares different diagnostic methods for HBoV1 infection.

In conclusion, we did not find a correlation between HBoV1 genome load and mRNA detection or serology in adeno-/tonsillectomy patients. Our findings support the use of HBoV1 mRNA detection and serology as more specific diagnostic tools to identify acute bocavirus infection.

NOTES

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Potential conflicts of interest

Dr. Allander has a patent Human bocavirus and methods of diagnosis and treatment licensed to Karolinska Institutet Innovations AB. Other authors: no reported conflicts.

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Table 1. Adenotomy patients with HBoV1 DNA-positive NPA samples

Case no.	Age (y)	Adenotomy indication	Symptoms[a] on the operation day	HBoV1 NPA PCR result	HBoV1 DNA load (cp/ml) in NPA	mRNA NPA
B013	4	ROM	yes	pos	128800	neg
B038	3	ROM	no	pos	123800	neg
B061	2	ROM	no	pos	550000	neg
B064	2	ROM	no	pos	176800	neg
B066	3	ROM	yes	pos	141200	neg
B073	3	ROM	no	pos	100396800	neg
B074	5	ROM	no	pos	36200	neg
B087	3	ROM	yes	pos	28269400	neg
B100	6	AH	yes	pos	19708800	neg
B122	8	AH	yes	pos	358200	neg
B126	4	AH	yes	pos	16800	neg
B129	2	ROM	yes	pos	117400	neg
B182	2	ROM	no	pos	20537600	neg
B184	1	ROM	no	pos	2227000	neg
B194	2	ROM	yes	pos	91600	neg

Abbreviations: y, years; ROM, recurrent otitis media; AH, adenoid hypertrophy; NPA, nasopharyngeal aspirate; cp, copies. [a]One or more of the following: mild rhinitis, cough, symptoms of otitis, throat pain, upper airway obstruction symptoms.

305 **Table 2. Adeno-/tonsillectomy patients with HBoV1 DNA-positive NPA and/or tonsillar tissue**

306 **samples**

307

Case no.	Age (y)	Tonsillectomy indication	Symptoms[a] on the operation day	HBoV1 PCR result, NPA	HBoV1 DNA load (cp/ml), NPA	HBoV1 PCR result, tonsils
B004	6	ATH	no	pos	NA	neg
B008	6	ATH	no	pos	400	neg
B015	8	ATH	no	pos	500	neg
B021	8	ATH	no	pos	4400	neg
B028	16	RT, TH	no	pos	NA	neg
B051	7	ATH	no	pos	133200	neg
B069	8	RT	no	pos	600	neg
B113	12	ATH	NA	pos	119200	neg
B130	5	ROM, ATH	NA	pos	30400	neg
B160	7	ATH	yes	pos	4000	neg
B162	6	ATH	no	pos	210600	neg
B169	7	RT	no	pos	7600	neg
B185	7	ATH	NA	pos	8600	neg
B018	22	RT	yes	neg	0	pos
B019	5	ROM, RT, TH	no	neg	0	pos
B036	4	ATH	NA	neg	0	pos
B135	3	ATH	no	neg	0	pos
B193	2	ATH	no	neg	0	pos
B195	9	ATH	no	neg	0	pos

B198	3	ROM, ATH, recurrent fever	yes	neg	0	pos
B056	5	ATH	yes	pos	307800	pos
B082	4	ATH	no	pos	32200	pos
B106	4	ATH	no	pos	220800	pos
B150	5	RT, ATH	NA	pos	92400	pos
B197	3	ATH	no	pos	202600	pos

Abbreviations: y, years; ATH, adenotonsillar hypertrophy; TH, tonsillar hypertrophy, ROM, recurrent otitis media; RT, recurrent tonsillitis; NPA, nasopharyngeal aspirate; cp, copies; NA, not available; abs., absorbance (cutoff ≥0.131).

[a] One or more of the following: mild rhinitis, cough, symptoms of otitis, throat pain, upper airway obstruction symptoms.

[b] Paired serum samples; no increase in IgG.

[c] No acute-phase serum sample available.

[d] HBoV2 IgG positive; may influence induction of HBoV1 IgG through original antigenic sin [13].

[e] Very low absorbance level; 0,147. Together with a stable IgG level in paired samples, the interpretation is recent but non-acute HBoV1 infection.