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Human bocavirus in children: Mono-detection, high viral load and viraemia are associated with respiratory tract infection

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ABSTRACT

Background and objectives: Human bocavirus 1 (HBoV1) has recently been detected in children with respiratory tract infections (RTI). In order to study whether HBoV1 can cause RTI, we investigated its presence in children with upper RTI (URTI), lower RTI (LRTI) and a control group of children without RTI.
Study design: Nasopharyngeal aspirates (NPA) and blood samples were collected from children admitted to hospital with RTI from 6 June 2007 to 28 February 2009 ($n = 1154$), and from children admitted for elective surgery who had no RTI ($n = 162$). Using polymerase chain reaction (PCR), the NPAs were examined for 17 infectious agents including HBoV1. Blood samples were tested with HBoV1-PCR only.
Results: HBoV1 was detected in NPAs from 10% of patients and 17% of controls. Adjusted for age, gender and the presence of other viruses, HBoV1 was not associated with RTI. In the HBoV1-positive NPAs, at least one other virus was detected in 75% and the virus appeared alone in 25%. Adjusted for age and gender, the detection of HBoV1 as the sole virus was associated with RTI, but not with LRTI. Viraemia was found only in children with RTI. The study showed that it was associated with RTI and LRTI. A high HBoV1-load was associated with LRTI, but not with RTI. No interactions between HBoV1 and other infectious agents were found.
Conclusions: Our data support the hypothesis that HBoV1 causes RTI in children, because detection of HBoV1 alone, viraemia and high viral load are associated with RTI and/or LRTI in this age group. However, HBoV1 is common in healthy children.

1. Background and objectives

The human bocavirus, belonging to the family *Parvoviridae* and the genus bocavirus, was discovered in 2005.[1] Four different species of human bocavirus have been proposed, and the name human bocavirus 1 (HBoV1) has now been suggested for the originally discovered virus.[2] It has been associated with respiratory tract infections (RTIs) in children and a link to more complex clinical conditions in immunosuppressed patients has been suggested.[3–5] However, HBoV1 is frequently found together with other respiratory viruses and consequently its causative role in childhood RTI may be questioned. Possible interactions between HBoV1 and other viruses have also been discussed.[6] We studied the role of HBoV1 in childhood RTI by comparing its presence in children hospitalised with RTI with a control group of symptom-free children admitted to elective surgery. We also looked for several other respiratory pathogens in order to evaluate their coexistence with HBoV1.

2. Study design

The study was performed at the Department of Paediatrics, St. Olavs Hospital, Trondheim University Hospital during the period 6 June 2007 to 31 August 2009. St. Olav's Hospital is a regional hospital for mid-Norway covering a population of 640 000. As part of routine clinical work at our department, and on the discretion of the medical doctors, nasopharyngeal aspirates (NPAs) were collected from most children who were admitted with RTI. The parents were informed about the study and asked to participate. In addition to the NPA, a blood sample for the study was collected simultaneously with routine blood samples from included children.

The children were classified as having either lower or upper respiratory tract infection (LRTI; URTI). LRTI was diagnosed in the presence of dyspnoea, signs of lower airway obstruction (wheezing, retractions) and/or a positive chest X-ray (infiltrates, atelectasis and

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air trapping). URTI was diagnosed when rhinitis, pharyngitis and/or otitis media were found without signs of LRTI.

A control group ($n = 162$) was included prospectively in the same time period. The controls were children admitted for elective surgery who had no symptoms of RTI in the last 2 weeks. Every week throughout the study period we asked the parents of 2–4 children to participate. Most controls had surgery for cryptorchidism, hernia repair or benign skin tumours, and none for ear, nose and throat surgery. A total of 1316 samples were included in the study, 1154 from patients and 162 from controls. The mean ages in the patient and control groups were 35 and 43 months ($p = 0.05$).

Using polymerase chain reaction (PCR), the NPAs were tested for adenovirus, HBoV1, coronavirus (OC43, 229E and NL63), enterovirus, human metapneumovirus, influenza A and B virus, parainfluenza virus type 1–3, RS-virus (RSV), rhinovirus, *Bordetella pertussis, Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*. Nucleic acid was extracted with NucliSens®easyMag® (bioMerieux). All PCRs were in-house real-time assays based on TaqMan probes. The analyses were carried out as part of the daily laboratory routine and mainly performed within 24 h after sample collection. The target for the HBoV1-PCR was the NP-1 gene; the primers and probe have been previously described.[7] Quantitative standards for the real-time HBoV1-PCR assay were made by cloning a plasmid (pCR®4-TOPO®) containing the PCR product. The amount of nucleic acid was measured and serial dilutions covering a range of seven logs were made. The viral load in each sample was recorded semi-quantitatively and grouped in three categories: high viral load (10^6–10^{10} copies ml^{-1}), medium viral load (10^4–10^6 copies ml^{-1}) and low viral load (10^3–10^4 copies ml^{-1}). The reportable range of the assay was from 1000 copies ml^{-1} (20 copies per reaction) to 10^{10} copies ml^{-1}. In addition, plasma samples available from 60 of the 144 HBoV1-positive children were examined with the HBoV1-PCR.

All NPAs were collected in ordinary virus transport media without antibiotics and were cultured for viruses using standard cell lines. The transport media were also used to culture bacteria using standard agarose media. Growth of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catharralis* were recorded.

Plasma from a patient in whom HBoV1 had been detected in blood was diluted 1:1000 and pre-centrifuged at 16 000 × *g* for 30 min. 175 μl of the supernatant was then ultra-centrifuged at 27 psi (90 000 rpm) directly to 400 mesh carbonated formvar-covered copper grids and stained with 2% phosphotungstic acid. The grids were examined at magnifications 50 000× to 200 000× with Jeol JEM-1011 (Jeol Ltd.).

Statistical analysis was done by Chi-squared test for categorical data and Student's *t*-test for continuous data. Multiple logistic regression analysis was used to evaluate the association between HBoV1 and RTI, controlling for differences in age, gender and the presence of other viruses among cases and controls. We report the odds ratio (OR) with 95% confidence interval (95% CI) and the corresponding *p*-value as a measure of the strength of the association. All analyses were performed using SPSS software version 15 (Statistical Package of Social Science Inc.).

3. Results

In all, 144 of 1316 samples (11%) were positive for HBoV1. Fewer HBoV1-positive samples were found in the summer months, but the difference was not significant when adjusted for the number of samples received each month (Fig. 1). In total, 63% of the children were boys (61% in the patient group and 75% in the control group). The mean age of the HBoV1-positive patients was 19 months (SD: 10.6 months) and of the controls, 33 months (SD: 14.2 months) ($p < 0.001$). One-hundred and seventeen of 1154 samples (10%) from children with RTI were positive for HBoV1. Of these, 40 (37%) had URTI and 68 (63%) LRTI (of which 49% had bronchiolitis and 14% pneumonia). Of the 162 controls, 27 (17%) were positive for HBoV1. In a multiple logistic regression analysis adjusting for age, gender and the presence of other respiratory viruses, we found no association between a positive HBoV1-PCR test in NPA and RTI (OR: 0.8, 95% CI: 0.5–1.3, $p = 0.30$).

Among the 144 HBoV1-positive NPA samples, HBoV1 was detected alone in 25% (36 of 144). Twenty-nine percent (34 of 117) of the patient samples were positive for HBoV1 alone and 7% (2 of 27) of the controls ($p = 0.02$). One additional virus was found in 46%, two in 18%, three in 10% and four in 1% ($n = 144$). Rhinovirus, enterovirus, adenovirus and RSV were the most commonly co-detected viruses (Table 1). A logistic regression analysis on the HBoV1-positive samples, adjusted for age and gender, showed that detection of HBoV1 alone was associated with RTI (OR: 5.2, 95% CI: 1.1–24.4, $p = 0.04$) but not LRTI (OR: 0.6, 95% CI: 0.2–1.4 $p = 0.20$).

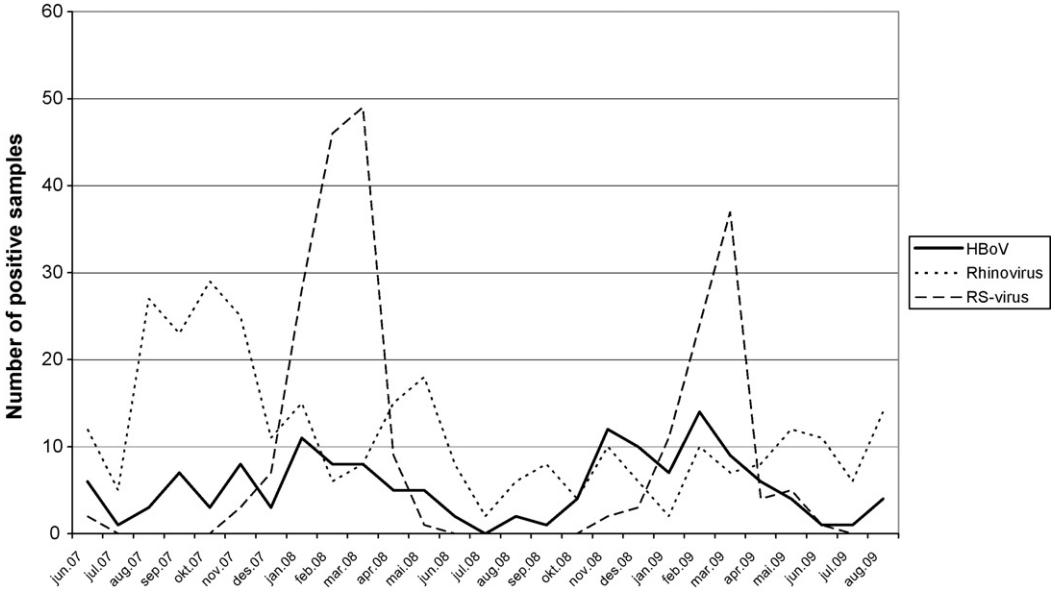

Fig. 1. Monthly distribution of samples positive for HBoV1, rhinovirus and RSV, in the period June 2007–August 2009.

Table 1
Viruses codetected in HBoV1-positive samples from patients with respiratory tract infection (RTI) ($n = 117$) and controls ($n = 27$).

Virus	Total		RTI patients		Controls	
	n	(%)	*n*	(%)	*n*	(%)
Adenovirus	30	(20.8)	21	(17.9)	9	(33.3)
Coronavirus 229E	1	(0.7)	1	(0.9)	0	(0)
Coronavirus OC43	7	(4.9)	6	(5.1)	1	(3.7)
Coronavirus NL63	2	(1.4)	1	(0.9)	1	(3.7)
Enterovirus	39	(27.1)	25	(21.4)	14	(51.9)*
Influenzavirus A	1	(0.7)	1	(0.9)	0	(0)
Influenzavirus B	1	(0.7)	1	(0.9)	0	(0)
Metapneumovirus	5	(3.5)	5	(4.3)	0	(0)
Parainfluenzavirus type 1	1	(0.7)	1	(0.9)	0	(0)
Parainfluenzavirus type 2	0	(0)	0	(0)	0	(0)
Parainfluenzavirus type 3	6	(4.2)	6	(5.1)	0	(0)
Rhinovirus	42	(29.2)	32	(27.4)	10	(37.0)
RS-virus	23	(16.0)	23	(19.7)	0	(0)*

* $p < 0.05$.

A high viral load (>10^6 copies ml^{-1}) in the NPA was found in 33% (39 of 117) of patients and 15% (4 of 27) of controls. However, when we adjusted for age, gender and presence of other viruses, a high viral load was not associated with RTI (OR: 1.4, 95% CI: 0.4–5.1, $p = 0.57$). A very high viral load (>2×10^8 copies ml^{-1}), though, was clearly associated with RTI, as no controls and 14 patient samples had a copy number higher than this. LRTI was found in 82% (28 of 34) of the patients with a high viral load (>10^6 copies ml^{-1}) and in 54% (40 of 74) of patients with moderate or low viral load. (Nine children with complex clinical conditions were excluded from this analysis.) Adjusted for age, gender and other viruses, a high viral load was associated with LRTI (OR: 3.6, 95% CI: 1.2–10.7, $p = 0.02$).

HBoV1-viraemia was found in 45% of patients with available samples (18 of 40) and in none of the controls (0 of 20). Viraemia was almost exclusively detected in patients younger than 2 years (16 of 18). More children with LRTI (57%, 16 of 28) than URTI (20%, 2 of 10) had viraemia ($p = 0.04$). (Two children with complex clinical conditions were excluded from this analysis.) Furthermore, viraemia was present more frequently in patients with a high viral load in NPA (70%, 14 of 20) compared to patients with a moderate or low viral load (10%, 4 of 40) ($p < 0.001$). Thirty-three percent (6 of 18) of the patients with viraemia had HBoV1 alone in the NPA, compared to 10% (4 of 42) of the patients without viraemia ($p = 0.052$, Fisher's exact test). Rhinovirus (33%, 6 of 18) was the most commonly co-detected virus among the viraemic patients.

Electron microscopy examination of blood from a boy of 18 months with bronchiolitis, HBoV1 alone and a high HBoV1-load in NPA and blood, showed viral particles with size ~25 nm, compatible with human bocavirus (Fig. 2).

Adenovirus and enterovirus were more frequent in the HBoV1-positive NPA samples than the negative ones (Table 2). In contrast, two other commonly detected viruses, rhinovirus and RSV, were as common in the HBoV1-positive as the negative ones. The frequency of RTI among children positive for rhinovirus, RSV, enterovirus or adenovirus was not affected by the simultaneous presence of HBoV1.

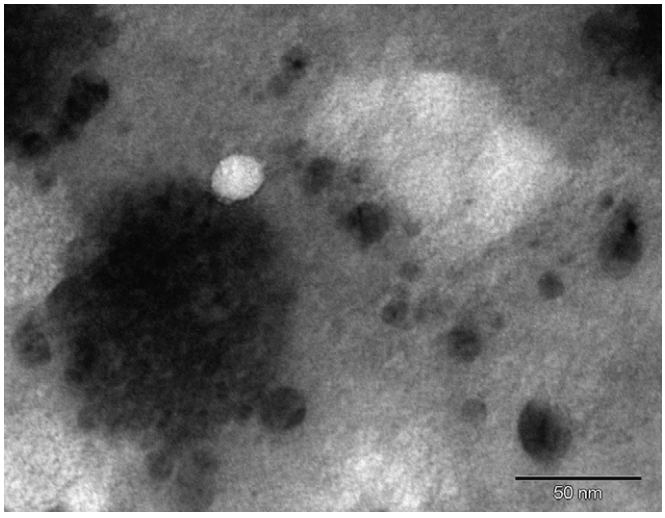

Fig. 2. Electron microscopic image taken of serum from a boy of 18 months with HBoV1-infection showing a viral particle with size ca. 25 nm. The image is taken at 200 000× magnification.

When bacteria were considered in addition to viruses, multiple detection was made in 85% of the HBoV1-positive NPA samples. One patient had *M. pneumoniae*. *S. pneumoniae*, *H. influenzae* and *M. catharralis* were present in 26%, 19% and 27% of the HBoV1-positive samples, respectively. There were no differences in the distribution

Table 2
The four viruses most commonly co-detected with HBoV1 distributed by study group and whether HBoV1 was present ($n = 144$) or not ($n = 1172$).

		Total population ($N = 1316$) *n* (%)[a]		Patient group ($N = 1154$) *n* (%)[b]	Control group ($N = 162$) *n* (%)[b]	
Adenovirus	HBoV1 present	30 (20.8)	$p < 0.001$	21 (70.0)	9 (30.0)	$p = 0.19$
	HBoV1 not present	91 (7.8)		74 (81.3)	17 (18.7)	
Enterovirus	HBoV1 present	39 (27.1)	$p < 0.001$	25 (64.1)	14 (35.9)	$p = 0.53$
	HBoV1 not present	111 (9.5)		78 (70.3)	33 (29.7)	
Rhinovirus	HBoV1 present	42 (29.2)	NS	32 (76.2)	10 (23.8)	$p = 0.17$
	HBoV1 not present	266 (22.7)		225 (84.6)	41 (15.4)	
RSV	HBoV1 present	23 (16.0)	NS	23 (100.0)	0 (0.0)	$p = 0.41$
	HBoV1 not present	207 (17.7)		201 (97.1)	6 (2.9)	

[a] Percentage of corresponding virus within the two groups either positive ($n = 144$) or negative ($n = 1172$) for HBoV1.
[b] Percentage within row showing the distribution of the co-detected virus between patient group and control group.

of bacteria among the HBoV1-positive and negative samples, or between patients and controls (data not shown).

4. Discussion

Our study confirms that HBoV1 is frequently found in children with RTI, and often simultaneously with other respiratory viruses. In contrast to most other studies, we also detected HBoV1 in many children without RTI.[3,8–10] Nevertheless, our findings indicate that HBoV1 causes disease, because detection of the virus alone, a high viral load in NPAs and viraemia were associated with RTI in hospitalised children.

HBoV1 was detected alone in a third of the patients but only in a few of the controls, and even if the majority in both groups had multiple viruses, this finding supports a causal role of HBoV1 in relation to RTI. HBoV1 alone was not associated with a higher occurrence of LRTI. This finding has been reported previously.[9,11] We did not control for duration of symptoms before admission, and this may be an explanation because duration of symptoms and disease severity are likely to be related.

We were surprised to find no association between a high viral load ($>10^6$ copies ml^{-1}) in the NPAs and RTI. This may also be due to the fact that we did not control for duration of symptoms before admission. However, an even higher viral load ($>2 \times 10^8$ copies ml^{-1}) was seen only in children with RTI. Furthermore, an association was found between a high viral load ($>10^6$ copies ml^{-1}) and infection in the lower respiratory tract. This finding may indicate that high viral load is associated with more severe disease, and may represent a dose-effect argument for a causal relation between HBoV1 and RTI.

The detection of HBoV1-DNA in plasma may represent viraemia or simply leakage of DNA from infected cells. Fig. 2 shows the presence of viral particles in blood from one patient. This may suggest that detection of HBoV1-DNA in blood represents a true viraemia, but this singular finding should be studied on a larger scale. Nevertheless, as it has been shown before,[9,12] HBoV1-viraemia was strongly associated with RTI in general and, although weaker, with LRTI. It is most likely that a viraemia with HBoV1 indicates a present HBoV1-infection.

Another characteristic feature of HBoV1-viraemia in our study was that it was almost exclusively seen in children of less than 2 years, and thus may be a marker for primary HBoV1-infection. Serological studies show a correlation between IgM-response, IgG-seroconversion and an episode of HBoV1-viraemia related to RTI in small children.[11,12] At the age of three, 70–90% of children are seropositive for HBoV1,[11,13] suggesting that the infection in early childhood gives immunity against later infections. Most patients with viraemia, high viral load and LRTI in our study were in the 12–17 months age group, the age at which most children have lost the protection of maternal antibodies. In the older children, however, the majority of the HBoV1-detections could represent asymptomatic re-infections, reactivations of latent/persistent infections or long-time virus shedding. Reactivation of other human pathogenic parvoviruses is known to happen.[14,15] Further studies on this issue are needed.

Most previous studies detected HBoV1 among only a few controls,[3,8–10] but two reported similarly high rates to ours of HBoV1-positive samples in healthy children.[16,17] The major strengths of our study are the large case group and the prospective inclusion of controls from the same time period and same geographical area, and the use of the same sampling technique and test algorithm for both groups. Patients admitted for ear, nose and throat surgery were not included in the control group because many of these diseases may be related to viral infections. The main differences between cases and controls that might influence the evaluation of HBoV1 were age, gender and the presence of other viruses. In the analyses, we used multiple logistic regression analysis to adjust for these factors.

We detected multiple viruses in three quarters of the nasopharyngeal samples, as have other PCR-based studies.[9,18] Adenovirus and enterovirus were more common in children with HBoV1. Enterovirus can be detected in samples from patients several weeks after infection,[19] and shedding of HBoV1 over long periods has recently been documented.[17,20] Thus, HBoV1 and enterovirus seem to share a common tendency for prolonged shedding, which may explain their frequent co-detection. A criterion of 2 weeks without respiratory symptoms before inclusion in the control group is, in this context, relatively short. Long-time virus shedding is therefore a plausible explanation for the high occurrence of HBoV1 in our control group. It has previously been shown that strong immune stimuli can cause reactivation of adenovirus in adenoid tissue,[21] and therefore we speculate that the association between HBoV1 and adenovirus may be associated with reactivation of the latter. Reactivation of HBoV1, an alternative or supplementary explanation, has yet to be demonstrated. As expected, RSV was common in children with RTI and rare among controls, appearing at similar rates in children with or without HBoV1, which indicates the dominance of RSV. Similarly, for each of the other viruses studied, co-detection of HBoV1 did not increase the frequency of RTI (Table 2). Cultures for bacteria were included in the study in order to look for interactions with viruses, but no such interactions were seen for HBoV1. Therefore, our findings do not support the existence of significant interactions between HBoV1 and other respiratory viruses or bacteria. However, more studies are needed to clarify these complex matters.

Conflict of interest

None.

Funding

None.

Ethical approval

Approved by the Regional Committee for Medical and Health Research Ethics in Mid-Norway.

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