

# Association of Human bocavirus with Respiratory Infections in Outpatients and in Patients Attended at a Reference Hospital

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**Abstract** The role of Human bocavirus (HBoV) in human infectious disease is unclear due to the frequent detection of this virus in association with other respiratory viruses with a recognized pathogenic role in acute respiratory infection. We have analyzed the impact of HBoV in outpatients and in patients requiring hospitalisation or emergency attention for acute respiratory infections. Respiratory viruses were investigated by real-time PCR, direct antigen detection and/or viral culture by shell-vial assay. Nasopharyngeal aspirates, BAL and/or sputum samples from patients attended at a reference hospital, and nasal/throat swabs from outpatients were used. Respiratory viruses were detected in 660 samples (47%). HBoV detection rate was 12.6%, only preceded by respiratory syncytial virus (25%). Co-detections were observed in 12.9% of samples, and HBoV was present in 81% of them. Similar detection rates of HBoV were obtained in individuals with positive and negative results for other respiratory viruses (12.5% and 12.7%, respectively). The crossing point value was taken as a measure of HBoV viral load. Higher HBoV loads were observed in children, and in patients from the hospital. HBoV viral load was not associated with

symptoms of upper respiratory tract infection or lower respiratory tract disease. Although HBoV is frequently detected in respiratory specimens, there is a poor association between HBoV-positive specimens and clinical parameters. A clinical impact of HBoV in respiratory infection probably occurs in few cases.

**Keywords** Human bocavirus · Respiratory tract infection · Lower respiratory tract · Viral load

## Introduction

Human bocavirus (HBoV) is a parvovirus, distantly related to parvovirus B19. HBoV was first detected in nasopharyngeal aspirates from patients with acute respiratory infection (ARI) [1]. Recently, it has also been identified in blood and in fecal samples from patients with gastroenteritis [2, 7, 20, 25]. However, the role of HBoV in human infectious disease is unclear due to the frequent detection of this virus in association with other respiratory viruses (RV) with a recognized pathogenic role in ARI. Indeed, like other members of the family (e.g. Parvovirus B19), HBoV can be shed asymptomatically during a prolonged period [23]. Some studies have suggested that the determination of HBoV load in respiratory specimens could be useful for clarifying the role of HBoV in acute respiratory infections (ARI) [7, 23].

Most studies have investigated the role HBoV in ARI in the context of hospitalised patients. There are few studies that included control groups of asymptomatic individuals [2, 7, 11, 16, 26]. Fry et al. [7] showed that HBoV was more frequently detected in outpatients with influenza-like illness and hospitalised patients (4–5%) than in asymptomatic subjects (1%).

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Our laboratory is the regional laboratory in Andalusia belonging to the National Laboratory Network in Spain for Influenza Surveillance. Thus, each influenza season, we receive nasal/throat swabs from outpatients with symptoms of ARI, collected by 26 sentinel physicians from the Influenza Surveillance Network. The laboratory belongs to a reference hospital, which attended a population of 444,647 individuals in 2006 [24]. Respiratory specimens from individuals with ARI, hospitalised or attended at the Emergency Unit are also processed in the laboratory for the investigation of respiratory viruses (RV).

The aim of this work was to investigate the impact of HBoV in respiratory infections in patients attended at the hospital and in outpatients attended at health care centers.

## Materials and Methods

### Study Setting

The study period was comprehended from October 2006 to September 2008. Respiratory specimens from hospitalised patients or attended at the Emergency Unit (HP) for ARI, were sent to the laboratory within 1 h after collection. Nasal/throat swabs from outpatients' (OP) were collected in viral transport medium (supplemented with 1% bovine serum albumin) within two Influenza seasons (October to May of 2006–2007 and 2007–2008). Samples were sent to the laboratory at 4°C within 6 h.

### Laboratory Procedures

Routine virologic investigation in respiratory specimens from HP included: antigen detection techniques for respiratory syncytial virus (RSV) and influenza viruses (FLU); simultaneous culture in Hep-2, MDCK y LLC-MK2 cell lines using shell-vial assay (SV) [18] for RSV, FLU, parainfluenza virus (PIV) 1–3 and adenovirus (ADV) detection; and traditional tube culture (TC) in MRC-5 for rhinovirus and enterovirus detection [8]. In specimens from OP, SV with MDCK, followed by RT-PCR on the cell culture supernatant for detection and typing of FLU was carried out [22].

HBoV, human metapneumovirus (hMPV) and human coronaviruses (HCoV) were retrospectively investigated from samples previously frozen at  $-80^{\circ}\text{C}$ . Nucleic acids extraction was carried out from 200  $\mu\text{l}$  of samples using Cobas Ampliprep Total Nucleic Acid Isolation Kit and the instrument COBAS Ampliprep (Roche Diagnostics, Mannheim, Germany).

Real-time PCR for HBoV detection was carried out with the LightCycler FastStart DNA Master<sup>PLUS</sup> Hybridization Probes kit (Roche Diagnostics), and 0.5  $\mu\text{M}$  of primers and

0.2  $\mu\text{M}$  of taqman<sup>®</sup> probe targeted at the HBoV NP-1 gene [14]. The amplification was performed in a LightCycler 2.0 Instrument (Roche Diagnostics):  $95^{\circ}\text{C}/10$  min and 50 cycles of  $95^{\circ}\text{C}/8$  s +  $60^{\circ}\text{C}/20$  s. A single fluorescence reading was taken in each cycle. Readout was performed in channel 530.

For hMPV and HCoV detection, cDNA was synthesized from 10  $\mu\text{l}$  of nucleic acids using iScript<sup>TM</sup> Reverse Transcriptase System (Bio-Rad Laboratories, Hercules, CA). Real-time PCR for hMPV was carried out using primers and taqman<sup>®</sup> probe targeted at a fragment of the hMPV N gene [15]. Real-time PCR for HCoV detection (OC43, 229E, NL63 and HKU1) used primers and taqman<sup>®</sup> probes described by Kuypers et al. [12]. The same conditions described for HBoV real-time PCR were used. HKU-1 and OC43 were subsequently differentiated by a conventional PCR assay [13].

Primers and probes were synthesized by Applied Biosystems (Foster City, CA, USA).

### Statistical Analysis

Data were analyzed using the SPSS 15.0 software (SPSS Inc., Chicago, IL). Demographic and clinical data were collected from each patient: age, sex, symptoms of ARI and clinical criteria of influenza-like illness according to the ICHPPC-2 definition [4] from all OP, and the presence of lower respiratory tract diseases (LRTD, pneumonia, bronchiolitis, bronchitis, and acute exacerbation of asthma) from HP with HBoV-positive results. Along with descriptive analysis, bivariate analysis was carried out to compare clinical and epidemiological data with virological results using chi-square test. A  $P < .05$  was considered significant.

## Results

### Demographic, Epidemiologic and Clinical Characteristics

A total of 1,400 specimens were analyzed: 910 nasopharyngeal aspirates, 457 nasal/throat swabs, 20 sputum samples and 13 bronchoalveolar lavages. By sex, 674 (48.1%) of patients were males, and 726 (51.9%) were females. Patients' ages (available from 1,189 individuals) ranged between 0 and 90 years old (median age: 2 years).

RV were detected in 660 specimens (47.1%): 171 FLU (12.2%; 103 FLU A and 68 FLU B), 177 (12.6%) HBoV and 70 (5%) hMPV. RSV, ADV, PIV 1–3, rhinovirus and enterovirus were detected in 240 (25%), 20 (2%), 19 (1.9%), 9 (0.9%) and 3 (0.3%) samples from HP, respectively. HCoV were investigated in 475 specimens and 35 cases were positive (13 OC43, 5 229E, 14 NL63 and 3

HKU-1). The distribution of the most frequently detected viruses by origin, age groups and year periods is shown in Table 1. The comparison of HBoV detection rates by origin yielded no significant differences between HP and OP, whereas hMPV was detected more frequently in HP. FLU detection rates were significantly higher in OP, but this result is biased because more FLU viruses are annually detected within the context of the Influenza Surveillance Network. Indeed, HBoV detection rates were not statistically different comparing both age groups, whereas RSV and hMPV were detected mainly in paediatric population.

#### Co-detections

Co-detections were observed in 85 subjects (12.9% of positives): 19 (27%) hMPV-positive cases, 26 (10.8%) RSV-positive cases, and 28 (16.4%) FLU-positive cases; HBoV was the virus co-detected in 78.9% (15 cases), 80.8% (21 cases) and 75% (21 cases), respectively. Other co-detections were: 4 HBoV + ADV, 3 HBoV + HCoV, 2 HBoV + FLU + HCoV, 1 HBoV + PIV-2, 1 HBoV + RSV + HCoV, 1 HBoV + hMPV + PIV-3, 6 HCoV + RSV, 4 HCoV + FLU, 3 hMPV + RSV, 1 hMPV + PIV-3, 1 RSV + ADV, and 1 FLU + ADV.

#### Role of HBoV in Respiratory Tract Infections

##### Qualitative Analysis

Since no control group had been included, to evaluate the role of HBoV in ARI, the detection rates of HBoV were compared in two population groups. Firstly, we excluded the presence of HBoV to divide the study population in two groups. One group comprehended cases positive for non-HBoV viruses ( $n = 552$ ) and the other group included cases negative for non-HoV viruses ( $n = 848$ ). Subsequently, HBoV detection rate was calculated in both groups. HBoV was detected in 69 (12.5%) and 108 cases

(12.7%) of cases positive and negative for non-HBoV viruses, respectively ( $P = 0.935$ ).

Similarly, other three different analyses were carried out with the most frequently detected viruses, i.e. RSV, hMPV and FLU. The presence of RSV, hMPV and FLU was independently compared in two population groups in each analysis: cases positive for non-RSV and negative for non-RSV viruses, cases positive for non-hMPV and negative for non-hMPV viruses, and cases positive for non-FLU viruses and negative for non-FLU viruses, respectively. Contrarily to the results obtained with HBoV, significant differences between both groups were observed in each analysis. RSV was detected in 8.3% and 21.4% of cases positive and negative for non-RSV viruses, respectively ( $P < 0.001$ ); hMPV was detected in 3% and 7% of cases positive and negative for non-hMPV viruses, respectively ( $P = 0.002$ ); and, FLU was detected in 6.7% and 15.5% of cases positive and negative for non-FLU viruses, respectively ( $P < 0.001$ ).

No relationship was found between HBoV detection and clinical symptoms in OP (Table 2); otherwise, fever, cough and ILI criteria were statistically higher in confirmed FLU cases.

#### Comparison of HBoV Loads with Demographic, Epidemiologic and Clinical Characteristics

Crossing point (Cp) value of the real-time PCR was used as an approximate measure of HBoV load. A previous validation of the assay was carried out. The intra-assay variability was calculated by testing ten times two different HBoV-positive specimens. Inter-assay reproducibility was calculated by testing ten times other two specimens in independent runs. The intra-assay coefficient of variation was 0.27% (Cp =  $26.08 \pm 0.069$ ) and 0.8% (Cp =  $34.43 \pm 0.30$ ) for both specimens. The inter-assay coefficient of variation was <1% (Cp =  $25.06 \pm 0.26$ ) and 1.5% (Cp =  $38.51 \pm 0.58$ ) for the other two samples tested.

**Table 1** Respiratory viruses most frequently detected in the study population [ $n(\%)$ ]<sup>a</sup>

Virus	By origin		<i>P</i>	By age groups <sup>b</sup>		<i>P</i>	By year period			<i>P</i>
	Hospital ( $n = 959$ )	Community ( $n = 441$ )		≤14 years ( $n = 865$ )	>14 years ( $n = 324$ )		Nov–Jan ( $n = 716$ )	Feb–Apr ( $n = 503$ )	May–Oct ( $n = 181$ )	
RSV	240 (25)	ND	–	149 (17.2)	4 (1.2)	<0.001	182 (25.4)	56 (11.1)	2 (1.1)	<0.001
HBoV	110 (11.5)	67 (15.2)	0.057	138 (16)	39 (12)	0.1	113 (15.8)	52 (10.3)	12 (6.6)	<0.001
FLU	47 (4.9)	124 (28.1)	<0.001	109 (12.6)	56 (17.3)	0.047	96 (13.4)	62 (12.3)	13 (7.2)	0.073
hMPV	59 (6.1)	11 (2.5)	0.003	58 (6.7)	3 (0.9)	<0.001	7 (1)	60 (11.9)	3 (1.7)	<0.001
Total <sup>b</sup>	456 (47.5)	202 (45.8)		454 (52.5)	102 (31.5)		398 (55.6)	230 (45.7)	31 (17.1)	

<sup>a</sup> No. positives (% respect to total patients in each column); ND not determined

<sup>b</sup> Age was known from 1,189 patients

**Table 2** Correlation between clinical symptoms of upper respiratory tract infection and detection of HBoV and FLU in outpatients

Data	HBoV [ <i>n</i> (%) <sup>a</sup> ]		<i>P</i>	FLU [ <i>n</i> (%) <sup>a</sup> ]		<i>P</i>
	Positive ( <i>n</i> = 67)	Negative ( <i>n</i> = 374)		Positive ( <i>n</i> = 124)	Negative ( <i>n</i> = 317)	
Fever ( <i>n</i> = 380)	58 (86.6)	322 (86.1)	1.0	119 (96)	261 (82.3)	<0.001
Cough ( <i>n</i> = 400)	59 (88.1)	341 (91.2)	0.491	121 (97.6)	279 (88.0)	0.001
Rhinorrhoea ( <i>n</i> = 363)	57 (85.1)	306 (81.8)	0.604	107 (86.3)	256 (80.8)	0.211
Sore throat ( <i>n</i> = 333)	51 (76.1)	282 (75.4)	1.0	99 (79.8)	234 (73.8)	0.218
Gastrointestinal symptoms ( <i>n</i> = 57)	8 (11.9)	49 (13.1)	1.0	21 (16.9)	36 (11.4)	0.118
ILI by ICHPPC-2 criteria <sup>b</sup> ( <i>n</i> = 204)	31 (46.3)	173 (46.3)	1.0	81 (65.3)	123 (38.8)	<0.001

<sup>a</sup> Percentage of clinical symptoms with respect to total positive and negative laboratory results

<sup>b</sup> Clinical criteria of ILI according to the ICHPPC-2 definition [17]

We took a cut-off Cp of 28 to divide the HBoV-positive population in those with a Cp ≤ 28 (high-HBoV-load cases) and those with a Cp > 28 (low-HBoV-load cases). A significantly higher percentage of children under 1 year gave a HBoV Cp ≤ 28. No nasopharyngeal swab or any other sample type had Cp ≤ 28, compared with 18.3% of nasopharyngeal aspirates. All samples from OP gave Cp > 28 compared with 17.3% of HP with Cp ≤ 28.

LRTD was reported in 72.5% of the HBoV-positive HP. From these, HBoV was detected alone in 31 cases (53.4%). No significant differences were observed in the Cp values between specimens in which HBoV was detected alone or codetected, nor by comparing the year periods or the presence of LRTD (Table 3).

No clinical symptoms from OP, associated with upper respiratory tract infection, correlated with higher HBoV loads.

**Discussion**

RSV was the most frequently detected virus in hospitalised children, in whom this virus is the main causative agent of bronchiolitis [21]. The higher detection rate of FLU in OP is probably due to the different method used in this population group. RT-PCR on the SV supernatants has been previously demonstrated to be the most sensitive for the detection of FLU [22].

Other classical RV such as PIV, ADV and rhinovirus, may have been under diagnosed by the SV method used. Routine investigation of these viruses included antigen detection and/or viral culture by SV. Our detection rate of these RV in HP is lower than the reported in other studies [3]. The methodology may have contributed to this difference. Many virologists are currently using molecular assays for RV detection, which are more sensitive than cell

**Table 3** Relationship between epidemiological and clinical data and estimation of HBoV DNA load by measure of the Cp value

Data	<i>n</i>	Cp ≤ 28 [ <i>n</i> (%)]	<i>P</i>	
Ages' groups	≤1 year	83	16 (19.3)	0.006
	1–3 years	31	2 (6.5)	
	4–14 years	24	0 (0)	
	>14 years	39	1 (2.6)	
Sample type	NPA	104	19 (18.3)	0.001
	NTS	70	0 (0)	
	Others	3	0 (0)	
Patient's origin	Hospital	110	19 (17.3)	<0.001
	Community	67	0 (0)	
No. viruses detected	HBoV	108	14 (12.9)	0.321
	HBoV co-detection	69	5 (7.2)	
Year period	Nov–Jan	113	11 (9.7)	0.255
	Feb–Apr	52	8 (15.4)	
	May–Oct	12	0 (0)	
LRTD	Yes	58	12 (20.7)	0.802
	No	22	4 (18.2)	

NPA nasopharyngeal aspirates, NTS nasal/throat swabs, others BAL and sputum, LRTD lower respiratory tract disease

culture. Indeed, molecular assays have allowed the detection of newly-identified RV that do not grow or grow poorly in cell culture, some of which have well-defined etiologic roles in respiratory tract infection, as occurs with hMPV. These methods have led to frequent co-detections [10]. For this reason, PCR methods for diagnosis of ARI may sometimes provide limited value to the information that the virologist gives to the clinician.

As occurs with other RV, HBoV might be asymptotically shed into the respiratory tract during a prolonged time [23]. Thus, a HBoV-positive result cannot not probably give the same information as the detection of other viruses such as RSV, hMPV and FLU in terms of clinical significance. Our results demonstrated that HBoV detection was independent of the presence of other respiratory virus, since the same detection rates of HBoV were observed in cases with and without other virus. And contrarily, respiratory viruses such as hMPV, RSV and FLU, recognized as causal agents of ARI were detected more frequently alone than with other virus. Other previous studies have compared the presence of HBoV in patients with ARI and control groups [7, 11, 26]. In one study [26], high HBoV detection rates were detected in children <1 year though no significant differences were observed between symptomatic and asymptomatic patients. In another study, HBoV was detected in children with pneumonia, but this clinical finding was associated more probable to codetection of other viruses [7]. Finally, Kesebir et al. [11], detected HBoV in patients with ARI and negative for RSV, FLU, PIV and ADV, compared to asymptomatic individuals. However, HBoV was investigated by PCR and the other viruses, by direct fluorescence assay; thus, these viruses could have been underdiagnosed because a less sensitive detection method was employed by the authors.

We could not demonstrate a clear association between HBoV detection and symptoms of ILI in OP, in contrast with FLU, which was significantly associated with fever, cough and ILI, as previously reported (Table 2) [6, 19]. Indeed, No significant differences were found in HBoV detection rate between OP and HP, although previous studies [7] have reported more HBoV cases in HP with pneumonia.

Semiquantitative analysis of HBoV load was further carried out to evaluate if higher loads could be correlated with a clinical significance of the presence of HBoV in respiratory samples, as other authors have previously reported [2, 9]. We established a cut-off Cp value of 28, which approximately corresponded to  $10^4$  copies/reaction [14]. Although we made some modifications to the method, since we demonstrated a good reproducibility, we assumed that the cut-off established, clearly differentiated between patients with higher and lower HBoV load.

No significant differences were observed in the Cp values between specimens in which HBoV was detected alone or codetected.

A significantly higher percentage of children under 1 year gave a HBoV Cp  $\leq 28$  (Table 3). These results are not surprising, since children have higher titers of RV. In fact, certain rapid tools for diagnosis of RV, such as antigen detection methods, are only useful in children, whereas their use in adult population, especially in immunocompromised patients, is not recommended [5]. Thus, higher HBoV load in respiratory specimens should be interpreted carefully in children.

No nasal/throat swab gave a Cp  $\leq 28$ , compared with 18.3% of nasopharyngeal aspirates. Indeed, all samples from OP gave Cp  $> 28$  compared with 17.3% of HP with Cp  $\leq 28$ . As nasal/throat swabs were the specimens taken from OP and nasopharyngeal aspirates from HP, we cannot rule out that a higher viral load in the latter can be due to a better sampling instead of a real pathogenic role of HBoV. Previous reports have assessed that the recovery rate of respiratory viruses in nasopharyngeal aspirates is better than in nasal and/or throat swabs [17].

Finally, HBoV were compared with clinical symptoms of ILI in OP, and HBoV loads with the presence or absence of LRTD in HBoV-positive HP. No clinical symptoms from OP, associated with upper respiratory tract infection, correlated with the presence of HBoV. LRTD was reported in 72.5% of the HBoV-positive HP. From these, HBoV was detected alone in 31 cases (53.4%). However, higher HBoV loads were not associated with the presence of LRTD (Table 3).

Thus, HBoV DNA is frequently detected in respiratory specimens. We could not demonstrate that the presence of HBoV and/or higher loads correlated with clinical symptoms of ILI or LRTD.

Whether HBoV must be routinely investigated in patients with ARI has to be defined. By the results obtained here, this analysis could be limited to certain cases in which the severity of the disease and/or the absence of any other respiratory pathogen might justify HBoV investigation.

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