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Infections and Coinfections by Respiratory Human Bocavirus During Eight Seasons in Hospitalized Children

Cristina Calvo,[1,2]* María Luz García-García,[1,2] Francisco Pozo,[3] Daniel Carballo,[2] Eduardo Martínez-Monteserín,[2] and Inmaculada Casas[3]

[1]*Department of Pediatrics, Severo Ochoa Hospital, Leganés, Madrid, Spain*
[2]*University Alfonso X el Sabio, Madrid, Spain*
[3]*Respiratory Virus and Influenza Unit, National Microbiology Center (ISCIII), Madrid, Spain*

The human bocavirus (hBoV) has been identified in respiratory infections in children in a large number of studies. Despite this, the pathogenic role of the HBoV is under discussion. The main objectives of the study were: to determine the incidence of HBoV in hospitalized children; to describe the main clinical features of the positive children; and to compare the data with those from other viral infections in the same population. A prospective study was performed between 2005 and 2013 including children up to 14-year old with respiratory infection admitted to the Severo Ochoa Hospital (Spain). Nasopharyngeal aspirates were taken from 3,275 patients and were tested for HBoV and other 15 respiratory viruses by RT-nested PCR. HBoV was detected in 319 patients (9.9%); 80 cases as a single pathogen, and 239 cases (75%) as coinfections with other viruses. The HBoV was the fourth most common virus detected, behind respiratory syncytial virus (39.8%), rhinovirus (30.6%), and adenovirus (15%). The most common clinical diagnosis, in cases that HBoV was detected as a single pathogen was asthma exacerbation followed by pneumonia. A seasonal distribution was shown, with higher positivity rates in December and January. Children affected by HBoV were older than children infected by other viruses. Differences in terms of clinical diagnosis were found, bronchiolitis diagnosis was lower compared with the other viruses, and HBoV was associated with diagnosis of pneumonia, with increased use of antibiotics (41.8%), and radiographic infiltrates (47%). These findings could suggest a pathogenic role of HBoV in respiratory infections in children under 14 years of age. ***J. Med. Virol. 88:2052–2058, 2016***. Published 2016. This article is a U.S. Government work and is in the public domain in the USA.

KEY WORDS: human bocavirus; respiratory infections; viral infections; coinfections

INTRODUCTION

Human bocavirus (HBoV), a DNA virus classified in the Parvoviridae family, was first described in 2005 [Allander et al., 2005], and has been detected in up to 18% of hospitalized children with respiratory diseases [Jartti et al., 2012]. However, it has also been found in children with mild infections [Martin et al., 2015] and in asymptomatic ones [García-García et al., 2008]. Up to 75% of the HBoV infections are coinfections with one or more viruses [Calvo et al., 2007]. A prolonged viral shedding has been described, about 75 days in outpatients [Martin et al., 2010], and up to 4.5 months in hospitalized children [Blessing et al., 2009], which probably explains HBoV detection in asymptomatic cases [von Linstow et al., 2008]. Although the pathogenic role of HBoV has been questioned, serologic diagnosis of HBoV has recently confirmed significant increases in IgG antibodies in children with pneumonia. These results support the idea that it is a true pathogen in respiratory tract infections in children [Korppi et al., 2010]. Unfortunately, this technique is not yet available in many laboratories and requires an invasive procedure for diagnosis.

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*Correspondence to: Cristina Calvo, Servicio de Pediatría, Hospital Severo Ochoa, Avda, Orellana, s.n. 28911 Leganés, Madrid, Spain. E-mail: ccalvorey@ono.com

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In this context, we would like to contribute to the knowledge of HBoV infections with a long-term prospective study. Our aim was to determine the incidence of the HBoV in hospitalized children, its epidemiological profile and to describe the main clinical features of the infected children. We also compared the clinical characteristics of single HBoV infections with other prevalent single viral infections in the same population, in an attempt to differentiate the characteristics of each infection.

PATIENTS AND METHODS

Clinical Assessment

The study population comprised all children between the first month of life and 14 years of age with a respiratory tract disease admitted to the secondary public hospital Severo Ochoa (Leganés, Madrid), between September 2005 and August 2013. The study was approved by The Medical Ethics Committee. Prior to sample collection, an informed consent was obtained from parents or legal guardians. All patients were evaluated by an attending physician. Clinical characteristics of patients were analyzed. During the hospital stay, and as part of the study, a physician filled out a study-questionnaire with the clinical data.

Upper respiratory tract infection (URTI) was diagnosed in patients with: rhinorrhea and/or cough and no signs of wheezing, dyspnea, crackles or bronchodilator use, with or without fever. Asthma was diagnosed on the basis of the National Asthma Education and Prevention Program guidelines [NAEPP, 2002]. All other episodes of acute expiratory wheezing were considered to be recurrent wheezing. Acute expiratory wheezing was considered to be bronchiolitis when it occurred for the first time in children aged less than 2 years. Laryngotracheobronchitis was associated with inspiratory dyspnea and wheezing. Laryngitis was associated with inspiratory dyspnea without wheezing. Cases with both focal infiltrates and consolidation in chest X-rays were, in the absence of wheezing, classified as pneumonia.

Virus Detection

Clinical specimens consisted of a nasopharyngeal aspirate (NPA) taken from each patient at admission (Monday–Friday). All clinical specimens were sent to the Respiratory Virus and Influenza Unit at the National Microbiology Center (ISCIII, Madrid, Spain), for virological investigation. NPAs were processed within 24 hr after collection. Upon receipt, three aliquots were prepared and stored at −70°C.

Polymerase Chain Reactions (PCR) Methods for Detection of 16 Respiratory Viruses

Three RT-nested PCR assays were performed to detect a total of 16 respiratory viruses. In these assays, the reverse transcription (RT) and first amplification round were carried out in a single tube using the Qiagen OneStep RT-PCR kit (Qiagen, Hilden, Germany). Influenza A, B, and C viruses were detected by using previously described primer sets only to amplify influenza viruses in a multiplex PCR assay [Coiras et al., 2003]. A second multiplex PCR was used to detect parainfluenza viruses 1–4, human coronaviruses 229E and OC43, enteroviruses and rhinoviruses (RV) [Coiras et al., 2004]. Presence of respiratory syncytial virus (RSV) A and B types, human metapneumovirus (hMPV), human bocavirus (HBoV), and adenoviruses were investigated by a third multiplex RT-nested PCR-BRQ method [Calvo et al., 2010].

Statistical Analysis

Values were expressed as percentages for discrete variables, or as mean and standard deviation for continuous variables. Clinical characteristics of patients with single infections associated to HBoV were compared with those associated with coinfections of HBoV with other respiratory viruses. Single HBoV infections were also compared with single infections by RSV, RV, and hMPV. Clinical characteristics and laboratory variables were compared using the Student t-test, the Mann–Whitney U-test, the χ^2 test, and Fisher's exact test. A two-sided value of $P < 0.05$ was considered statistically significant. Results were adjusted for age. All analyses were performed using the Statistical Package for the Social Sciences (SPSS), Version 21.0.

RESULTS

A total of 3,275 cases of respiratory diseases were analyzed during the study period. One or more viruses were detected in 76.5% of the samples. Multiple infections were present in 29% (726) of the positive cases. The most frequent identified virus was RSV (39.8%), followed by RV (30.6%), and adenovirus (HAdV); 15%. HBoV was the fourth in frequency and it was detected in 319 cases (12.7% of the positives cases). Figure 1.

Respiratory Infections Associated With HBoV Detection

Out of 319 cases, 80 (25%) were single detections, and 239 (75%) were detected in coinfection with other viruses, mainly RSV, RV, and HAdV (Fig. 1). Circulation of HBoV was higher in autumn (60% of cases between November and December).

Clinical characteristics of patients with respiratory episodes associated to single HBoV detections were analyzed (Table I).These childreńs group mean age was 21 months (standard deviation; 24 months). Fever was present in 68.8% of cases, and hypoxia in 52% of them. Recurrent wheezing or asthma was diagnosed in 58.8% of cases, followed by pneumonia (22.5%). X-ray infiltrates were present in 47% of the patients. Clinical cases associated to single HBoV

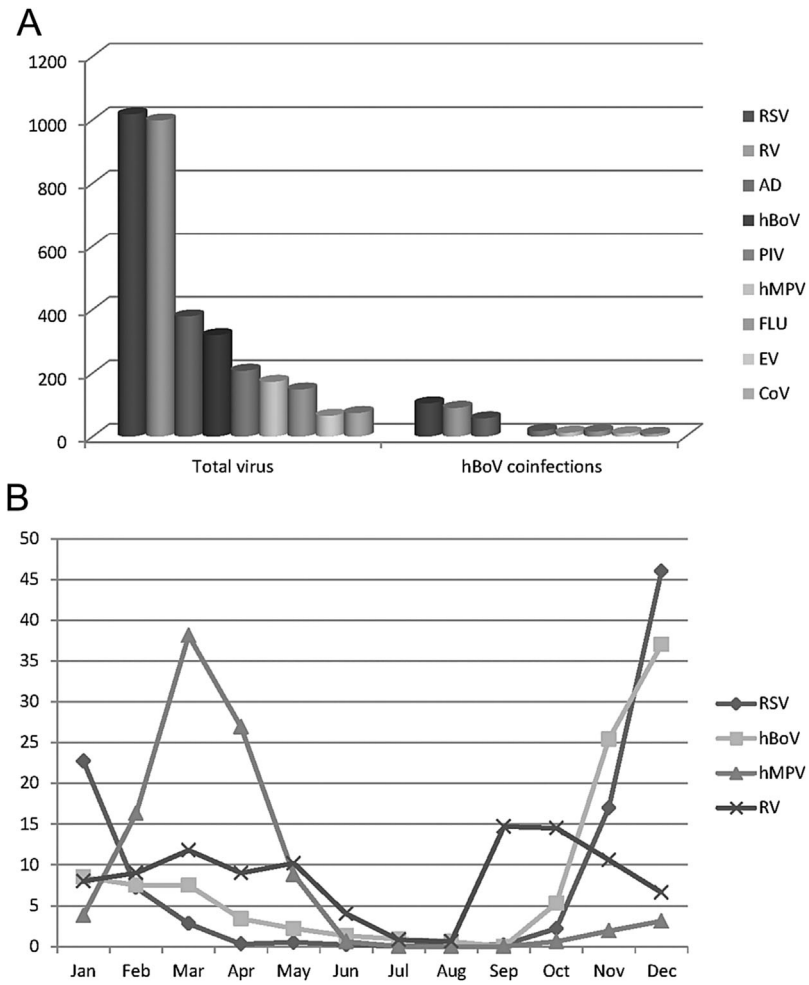

Fig. 1. **A**: Total viruses identified in the period of study, and virus detected in coinfection with hBoV. **B**: Monthly distribution of RSV, hBoV, hMPV, and RV.

TABLE I. Clinical Characteristics of Single HBoV Infections Compared With Coinfections of HBoV With Other Viruses

	Single HBoV (n = 80)	HBoV coinfections (n = 239)	*P*	OR (95%CI)
Males (%)	52 (65.8)	131 (54.6)	0.080	1.431 (0.951–2.154)
Age ± SD (months)	24.78 ± 24	20.6 ± 78.94	0.472	
Fever ≥ 38° (%)	55 (69.6)	178 (74.5)	0.398	0.836 (0.555–1.260)
Days of fever	3.04 ± 2.20	3.47 ± 3.40	0.280	
Sat O_2 < 95% (%)	41 (51.9)	146 (60.8)	0.162	0.762 (0.520–1.115)
Days of hypoxia	2.37 ± 1.53	2.40 ± 1.94	0.914	
Days of stay	3.74 ± 2.02	3.90 ± 2.33	0.559	
Chest X-ray (%)				
Infiltrate	37 (61.7)	105 (54.4)	0.322	
Normal	23 (38.3)	88 (45.6)		
Leukocytes ± SD cells/mm^3	15,620 ± 7,900	14,200 ± 6,000	0.219	
CRP mg/L	67 ± 79	49 ± 61	0.121	
Diagnosis (%)			<0.001	
Bronchiolitis	12 (15.6)	63 (27.5)		
Recurrent wheezing, or asthma crisis	47 (61)	143 (62.5)		
Pneumonia	18 (23.4)	17 (7.4)		
Antibiotic treatment (%)	33 (41.8)	80 (33.3)	0.174	1.308 (0.891–1.920)

OR, odds ratio; SD, standard deviation; CI, confidence interval; HBoV, human bocavirus; CRP, C-reactive protein.

detections were compared with the 239 cases of HBoV coinfections (Table I). Diagnosis was the only difference between groups. Cases with single HBoV detections were diagnosed as pneumonia more frequently ($P < 0.0001$) than coinfections. One patient with wheezing and with single HBoV detection had a concomitant urinary tract infection and a bacteremia.

Eight patients had two different admissions during the study period associated to HBoV (Table II), with an interval between clinical episodes of 15 days to 10 months. They were asymptomatic between episodes and we did not perform any viral determination during this period. One of these patients had two different admissions more than 2 years apart.

Comparison of Single HBoV Detections With Other Single Viral Infections

The clinical characteristics of the 80 patients with single HBoV detection were compared with the single infections associated with RSV, RV, and hMPV in the same period.

HBoV versus RSV. RSV single infections were present in 665 children and it was the most frequently identified virus. It was observed that HBoV affects more males (66% vs. 52%, $P = 0.02$) at a later age (25 vs. 9 months, $P < 0.0001$), and was associated with distinct clinical features. Episodes of wheezing or asthma and pneumonia were more frequent and bronchiolitis was less frequent and it was more characteristic in RSV infections ($P = 0.001$). Hypoxia (71% vs. 52%, $P = 0.001$), duration of hypoxia and days of stay were significantly higher in RSV infections. Leukocytes and C-RP were significantly higher in hBoV infections and also the proportion of patients treated with antibiotics (41% vs. 17%, $P < 0.001$). HBoV circulation (Fig. 1) was mainly in November and December (>60% of cases), coinciding with RSV (>60% in these 2 months). However, RSV was also frequently identified in January with 22.7% of its cases (Table III).

HBoV versus hMPV. hMPV single infections were present in 108 children during the study period. This group comprised children that were younger than HBoV-positive children (14 vs. 25 months, $P = 0.002$). Diagnosis of pneumonia was more frequent in HBoV children who also received more antibiotics (41% vs. 26%, $P = 0.026$). Leukocytes in blood and CRP were also significantly higher in HBoV group. Nevertheless, hospitalization was longer in the hMPV group ($P = 0.06$). hMPV was mainly detected in Spring (March–May, Fig. 1) (Table IV).

HBoV versus RV. RV single infections were present in 555 cases, and it was the second most prevalent virus. Both groups were of a similar age. The proportion of children with fever was higher in HBoV infections (68% vs. 41%, $P < 0.0001$). As with the other comparisons, diagnosis of pneumonia was more frequent in HBoV group and these children also had fever more frequently and received more antibiotics (41% vs. 20%, $P = 0.0001$). No other differences were found between them. RV circulated throughout the year, with a higher incidence in autumn (Fig. 1; Table V).

DISCUSSION

HBoV is, in our experience, the fourth virus identified in respiratory infections in hospitalized children. However, in only 25% of the cases it was detected as a single pathogen, with the remaining cases being characterized as coinfections. The single infections were characterized by the presence of high fever, wheezing, a significant percentage of pneumonia, hypoxia, moderate leukocytosis and elevated CRP, all of which lead to frequent antibiotic treatment. Children were on average 2-year old and infections occurred mainly in November and December. Although HBoV seasonally coincides with RSV, clinical characteristics of both viruses are quite different. RSV affects younger children, causing bronchiolitis and it is less frequently associated with radiographic

TABLE II. Patients With More Than One Sample Positive for HBoV

Case	Age	Sex	Date of admission	Fever	Hypoxia	Diagnosis	Coinfection	Virus
1	7 months	M	01.20.2006	No	No	Recurrent wheezing	No	
	17 months		11.16.2006	Yes	No	Recurrent wheezing	Yes	RSV, PIV
2	13 months	M	11.29.2010	No	No	Recurrent wheezing	Yes	AD
	13,5 months		12.10.2010	Yes	Yes	Recurrent wheezing	Yes	RV
3	6 months	M	02.09.2007	Yes	No	Bronchiolitis	No	
	41 months		12.23.2009	Yes	Yes	Recurrent wheezing	Yes	RSV
4	6 months	F	11.21.2010	No	Yes	Recurrent wheezing	Yes	PIV
	7 months		12.19.2010	No	yes	Recurrent wheezing	Yes	RV
5	38 months	M	12.23.2009	Yes	Yes	Pneumonia	Yes	RV
	41 months		03.21.2010	No	No	Pneumonia	No	
6	5 years	M	02.25.2011	Yes	Yes	Asthma crisis	No	
	5 years		04.05.2011	Yes	Yes	Asthma crisis	No	
7	10 months	F	01.01.2011	No	Yes	Recurrent wheezing	Yes	RSV
	12 months		03.08.2011	Yes	Yes	Recurrent wheezing	Yes	RV, hMPV
8	8 months	M	12.07.2011	No	Yes	Recurrent wheezing	No	
	9 months		12.29.2011	No	Yes	Recurrent wheezing	No	

HBoV, human bocavirus; RV, rinovirus; RSV, respiratory syncityal virus; hMPV, human metapneumovirus; AD, adenovirus; F, female; M, male.

TABLE III. Comparison of hBoV Single Infections With RSV Sngle Infections

	HBoV (n = 80)	RSV (n = 665)	*P*	OR (95%CI)
Sex, males (%)	53 (66.3)	350 (52.6)	0.021	1.666 (1.072–2.588)
Age ± SD (months)	25 ± 23.9	9.67 ± 12.83	<0.000	
Fever ≥ 38° (%)	55 (68.8)	424 (63.9)	0.388	1.217 (0.777–1.906)
Days of fever	3.04 ± 2.20	3.06 ± 2.83	0.943	
Sat O_2 < 95%	42 (52.5)	473 (71.2)	0.001	0.491 (0.326–0.741)
Days of hypoxia	2.33 ± 1.52	3.07 ± 2.31	0.044	
Duration of stay (days)	3.78 ± 2.02	4.71 ± 2.55	0.002	
Chest X-ray (%)				
Infiltrate	38 (62.3)	259 (49.9)	0.067	1.574 (0.963–2.574)
Normal	23 (37.7)	260 (50.1)		
Leukocytes ± SD cells/mm^3	15,620 ± 7,900	12,380 ± 7,800	0.014	
CRP mg/L	67 ± 79	28 ± 38	0.001	
Diagnosis (%)			<0.001	
Bronchiolitis	13 (16.7)	419 (63.4)		
Wheezing and asthma	47 (60.3)	212 (32.1)		
Pneumonia	18 (23.1)	19 (2.9)		
Antibiotic treatment (%)	33 (42.2)	117 (17.6)	<0.001	2.785 (1.853–4.186)

HBoV, human bocavirus; RSV, respiratory syncityal virus; OR, odds ratio; SD, standard deviation; CI, confidence interval.

TABLE IV. Comparison Between hBoV Single Infections and hMPV Single Infections

	HBoV (n = 80)	hMPV (n = 108)	*P*	OR (95%CI)
Sex, male (%)	53 (66.3)	62 (57.4)	0.219	1.246 (0.870–1.784)
Age ± SD (months)	25 ± 23.9	14.37 ± 20.85	0.002	
Fever ≥ 38° (%)	55 (68.8)	73 (67.6)	0.866	1.031 (0.720–1.478)
Days of fever	3.04 ± 2.20	2.75 ± 1.69	0.410	
Sat O_2 < 95%	42 (52.5)	68 (63)	0.150	0.784 (0.564–1.089)
Days of hipoxia	2.33 ± 1.53	2.72 ± 1.77	0.229	
Duration of stay (days)	3.78 ± 2.02	4.35 ± 2.10	0.062	
Chest X-ray (%)				
Infiltrate	38 (62.3)	43 (48.3)	0.091	1.407 (0.938–2.113)
Normal	23 (37.7)	46 (51.7)		
Leukocytes ± SD cells/mm^3	15,620 ± 7,900	11,700 ± 4,300	0.006	
CRP mg/L	67 ± 79	35 ± 41	0.019	
Diagnosis (%)			0.014	
Bronchiolitis	13 (16.7)	32 (29.9)		
Wheezing and asthma	47 (60.3)	65 (60.7)		
Pneumonia	18 (23.1)	9 (8.4)		
Antibiotic treatment (%)	33 (41.2)	28 (25.9)	0.026	1.462(1.057–2.021)

hBoV, human bocavirus; hMPV, human metapneumovirus; OR, odds ratio; SD, standard deviation; CI, confidence interval; CRP, C-reactive protein.

TABLE V. Comparison of Single HBoV Infections With Single RV Infections

	HBoV (n = 80)	RV (n = 555)	*P*	OR (95%CI)
Sex, Males (%)	53 (66.3)	334 (60.2)	0.298	1.258 (0.814–1.944)
Age ± SD (months)	25 ± 23.9	25.7 ± 28	0.832	
Fever ≥ 38° (%)	55 (68.8)	228 (41.1)	0.000	2.736 (1.752–4.275)
Days of fever	3.04 ± 2.20	2.36 ± 2.05	0.44	
Sat O_2 < 95%	42 (52.5)	284 (51.5)	0.873	1.034 (0.686–1.558)
Days of hipoxia	2.33 ± 1.53	1.95 ± 1.73	0.141	
Duration of stay (days)	3.78 ± 2.02	3.40 ± 2.15	0.130	
Chest X-ray (%)				
Infiltrate	38 (62.3)	149 (42.1)	0.003	2.014 (1.246–3.257)
Normal	23 (37.7)	205 (57.9)		
Leukocytes ± SD cells/mm^3	15,620 ± 7,900	20,800 ± 7,300	0.534	
CRP mg/L	67 ± 79	57 ± 92	0.644	
Diagnosis (%)			0.002	
Bronchiolitis	13 (16.7)	121 (22.8)		
Wheezing and asthma	47 (60.3)	316 (59.5)		
Pneumonia	18 (23.1)	58 (10.9)		
Antibiotic treatment (%)	33 (41.2)	111 (19.9)	0.000	2.411 (1.609–3.612)

OR, odds ratio; CI, confidence interval; SD, standard deviation; HBoV, human bocavirus; RV, rhinovirus.

infiltrates, it does not induce leukocytosis, and CRP is normal. hMPV circulate in spring, and clinical features are similar to those of RSV and different to HBoV infections. Rhinovirus circulates throughout the year, children have a similar age to the HBoV group, have also wheezing and infiltrates, but fever is less frequent. All these data suggest that HBoV infections have their own characteristics and support the role of this virus as an actual pathogen.

Although some authors [Martin et al., 2010] do not find differential clinical data in outpatients positive for HBoV, our series describes a set of characteristic clinical data in hospitalized children, different from single infections caused by other viruses as RSV or hMPV. A potential explanation for the differential clinical characteristics found in our patients might lie in the greater severity of hospitalized patients versus ambulatory ones. Furthermore, the number of patients analyzed by Martin could make it difficult to find distinguishing characteristics. Our series included a large number of patients and this allows us to draw conclusions on clinical data for a homogeneous population. Although there are very few studies comparing the clinical characteristics of HBoV infections with other viruses, some authors such as Moriyama et al. [2010] also find, clear differences between HBoV and RSV. Younger age, bronchiolitis, and hypoxia were more frequent in RSV group as is the case in our series. We have not found specific comparisons between HBoV and RV or hMPV, with the exception of previous work by our group [Calvo et al., 2007] and the study of Zhang in China with a small group of different viruses [Zhang et al., 2013]. They could not find any characteristic data of HBoV infection.

Many other studies have confirmed the relatively high frequency of HBoV infections in hospitalized children and their association with wheezing episodes [do Amaral de Leon et al., 2013] and pneumonia [Esposito et al., 2013; Wang et al., 2015]. Coinfections are as frequent as 75–80% also in previous studies [Calvo et al., 2007; Fry et al., 2007; Martin et al., 2010; Broccolo et al., 2015], and this might suggest that the symptoms may be caused by other viruses. In addition, HBoV is able to persist and therefore could be more often detected as an accompanying pathogen. Several authors have demonstrated that HBoV persist in different cells [Lüsebrink et al., 2011], intestinal tissues [Kapoor et al., 2011], lung and tumors [Schildgen et al., 2013], and in tonsillar tissue in children [Falcone et al., 2015]. Moreover, Byington et al. [2015] detected persistent HBoV in children, with and without symptoms, lasting longer than with other viruses. Therefore, the positivity of HBoV does not imply causality [Storch, 2015]. Furthermore, it is worth mentioning that Huang et al. [2012] and Dijkman et al. [2009] have shown that HBoV induces a cytopathic effect in human airway epithelial cell cultures, and it is very unlikely that a harmless bystander would be able to produce a cytopathic effect.

Prolonged HBoV shedding, found by Martin [2010] and others [Blessing et al., 2009], could also be the cause of detection in coinfection with other viruses, and their presence in asymptomatic children. More recently, in an outpatient population Martin et al. [2015], found that children infected with HBoV were more likely to experience recurrent cough episodes and to visit a healthcare provider during the 14 days following the time of initial detection of HBoV. Sequences of the samples from 12 of the 48 children that were positive for HBoV-1 were not 100% identical; and therefore, were considered to be HBoV reinfections that contributed to long-term shedding. Although HBoV sequences were not determined in our patients, these two facts could help to explain recurrent infections that we found in a percentage of patients in our series, which had more than one admission, with HBoV positive detection in a short period of time.

Although it might be expected that viral load could differ between acute infections or asymptomatic eliminations, quantification of viral load has been of no help to differentiate symptomatic from asymptomatic infections. In our opinion, this might happen because the technique for viral load measurement is not appropriate for respiratory secretions, where the standardization of the sample is difficult. This might also explain conflicting results in different studies [Martin et al., 2010; Ghietto et al., 2015; Principi et al., 2015]. Although not specifically included in this study, we ourselves have failed to find differences in viral load between symptomatic and asymptomatic patients. Still, severity of infection might be correlated to viral load in some studies as [Moesker et al, 2015] that reported a higher viral load in HBoV-positive patients admitted to an intensive care unit.

This study has some strength as its prospective nature, the long study period, the number of patients studied, both single and multiple detections, and the comparison with the most prevalent viruses. It also has some limitations, since we do not have a control group. However, we have confirmed in a previous work of our group, that symptomatic children in our setting have significantly higher HBoV positivity than healthy children [García-García et al., 2008]. We do not have serial samples collected to assess the shedding of HBoV. This prevents us from knowing if recurrent cases had a negative sample between episodes. We do not have serology and therefore, we cannot confirm that infections are acute and that HBoV is the causal agent. Serology has demonstrated that HBoV has a pathogenic role in respiratory infections [Kantola et al., 2008; Söderlund-Venermo et al., 2009; Don et al., 2010], but it has only been available in the last few years and only in some laboratories. It is remarkable, that in the study by Don et al. [2010], serology confirms the pathogenic role of HBoV in children with pneumonia, which is one of the most frequent diagnoses found in our

series. Other studies [Ghietto et al., 2015], match our series and find no clinical differences between single infections and coinfections with HBoV, which may also suggest the role of this virus in multiple infections.

In summary, we present a long prospective study of respiratory HBoV infections, with a considerable number of patients and compared with other viral infections, in which clinical features specific to this virus are shown. In the future, prospective studies, probably including both serologic studies and shedding of the virus, may help to clarify the remaining doubts about HBoV infections in children.

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