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ORIGINAL ARTICLE

Comorbidity and high viral load linked to clinical presentation of respiratory human bocavirus infection

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Abstract Human bocavirus (HBoV) is a new parvovirus associated with acute respiratory tract infection (ARTI). In order to evaluate HBoV significance as an agent of acute respiratory disease, we screened 1,135 respiratory samples from children and adults with and without symptoms during two complete calendar years. HBoV1 prevalence in patients with ARTI was 6.33 % in 2011 and 11.64 % in 2012, including neonatal and adult patients. HBoV1 was also detected in 3.77 % of asymptomatic individuals. The co-detection rate was 78.1 %. Among children, 87 % were clinically diagnosed with lower respiratory infection (no significant differences between patients with and without coinfection), and 31 % exhibited comorbidities. Pediatric patients with comorbidities were significantly older than patients without comorbidities. Patients with ARTI had either high or low viral load, while controls had only low viral load, but there were no clinical differences between patients with high or low viral load. In conclusion, we present evidence of the pathogenic potential of HBoV1 in young children with ARTI. Since patients with HBoV1-single infection are not significantly different from those with coinfection with respect to clinical features, the virus can be as pathogenic by itself as other respiratory agents are. Furthermore, an association between high HBoV1 load and disease could not be demonstrated in this study, but all asymptomatic individuals had low viral loads. Also, children with comorbidities are susceptible to HBoV1 infection at older ages than previously healthy children. Thus, the clinical presentation of infection may occur depending on both viral load and the particular interaction between the HBoV1 and the host.

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Introduction

Human bocavirus (HBoV) is a single-stranded DNA virus belonging to the family *Parvoviridae* (subfamily *Parvovirinae*) [1]. It has been associated with acute respiratory tract infection (ARTI), mainly in infants, which is a major cause of morbidity and mortality worldwide [2]. HBoV has been detected at variable rates from 0.9 % to 33 %, depending on the study population, usually involving children less than 5 years old with acute illness of the lower respiratory tract and wheezing [3]. HBoV has also been detected in adults [4, 5]. Our initial studies including hospitalized patients of a wide range of ages (but mostly pre-school aged children) showed that the prevalence of HBoV in the period 2007-2010 was ~20 % [4, 6]. However, it is difficult to evaluate the impact of HBoV infection on respiratory disease due to the detection of HBoV DNA in asymptomatic individuals and co-detection with other respiratory viral pathogens such as respiratory syncytial virus (RSV), adenovirus (AdV), parainfluenza virus (PIV), influenza virus (Flu) and metapneumovirus (MPV) [7–12]. High co-detection rates and the possibility of HBoV establishing a persistent infection [10, 13] have focused attention on HBoV viral load and clinical symptoms

[14–16]. Furthermore, while primary infection in infants has been linked to acute otitis media and respiratory illness [17, 18], HBoV reinfection events might be frequent in children [18]. HBoVs of three other species (HBoV2–4) have been discovered in stool samples [19–21], and all of them have been detected in the respiratory tract of children, although at a very low frequency [22]. The virus originally discovered by Allander et al. in 2005 is now named HBoV1 and is the one associated with respiratory illness [21].

The viral diagnostic panel generally used in hospitals and health centers typically includes RSV, AdV, PIV 1, 2 and 3, Flu A and B, and MPV. Thus, a high proportion (approximately 40 %) [23] of clinical samples analyzed using specific assays remain undiagnosed. Consequently, the aim of this study was to supply quality data to describe the clinical and epidemiological scenario of HBoV respiratory infection, in order to contribute to the evaluation of its significance as an agent of acute respiratory disease. We screened nasopharyngeal aspirates (NPA) and swabs (NPS) of children and adults with and without symptoms during two complete calendar years in order to gain information about general prevalence, seasonality, periodicity of epidemics, age distribution, viral load, major clinical manifestations, and circulating genotypes.

Materials and methods

Study population and clinical specimens

The study was performed with samples obtained from January 2011 through December 2012. The protocol was evaluated and approved by the Institutional Ethics Committee "CIEIS Polo Hospitalario del Niño y del Adulto, Córdoba", and all participants were included after informed consent. Respiratory specimens were collected from a total of 843 patients with ARTI. On the one hand, we processed NPA from 664 children ≤14 years old (300 patients from 2011 and 364 from 2012), admitted at the Children's Hospital of Córdoba City with acute infection of the lower airway tract. On admission at the hospital, complete physical examination data and the medical history of the children were properly obtained and recorded systematically. The diagnoses at admission of these pediatric patients were mainly bronchiolitis (48 %), pneumonia (40 %), and asthma exacerbation (10 %), among others (such as laryngitis and rhinitis). On the other hand, NPS from 179 adults aged 0 to 70 years old with upper and lower ARTI (79 patients from 2011 and 100 from 2012) were recruited from sentinel units of the program of surveillance of pneumonia in Córdoba, Argentina. All of the adult patients had influenza-type illness; 15 % of those attended to at the medical service were affected in the lower respiratory airway, and the remaining patients had upper ARTI. In addition, a group of 292 asymptomatic individuals of matching ages was sampled during the same time period. It consisted of healthy individuals who had not had ARTI within 2 weeks previous to the day on which the respiratory specimen was obtained (by NPS).

All clinical specimens were obtained by qualified personnel at the hospital room or at the locations of recruiting of controls and sent to the Institute of Virology within 24 h of collection, with adequate packaging for immediate sample processing and further virus detection.

Nucleic acid extraction and PCR screening

Nucleic acids were extracted from 100 µl of NPA or NPS specimens using guanidinium buffer and silica [4, 6]. Extracts were stored at −20 °C for subsequent HBoV detection. HBoV was detected by PCR as described previously [4]. PCR products were visualized in 8.5 % polyacrylamide gels stained with silver solution (0.11 M $AgNO_3$).

Relative quantification of HBoV-positive (HBoV+) specimens

Amplification was performed in an Applied Biosystems 7500 Real-Time PCR System, essentially as described elsewhere, using the NP1 gene as the target for relative quantification of HBoV1 [24]. The 25-µl amplification reaction contained 2.5 µl of DNA sample, 5 U of Platinum Taq DNA polymerase (Invitrogen) per µl, 0.04 µM each primer and 0.1 µl of a 1/100 SYBR Green (Invitrogen S-7563) dilution in DMSO. The viral load in each sample was estimated according to the 2^{-ddCt} method. The samples were grouped in two categories: low viral load (fold change, 0-49.9) and high viral load (fold change, ≥50).

Detection of common viral pathogens in HBoV-positive samples

HBoV+ samples from patients with ARTI were tested for the detection of other viral pathogens, including Flu A and B viruses, PIV1, −2 and −3, AdV, RSV and MPV by CDC hydrolysis probe-based quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). q-RT-PCR was carried out using AgPath-ID™ One-Step RT-PCR Reagents (Applied Biosystems), dual-labeled FAM-BHQ1 probes, and a pair of forward and reverse primers against Flu A, Flu B, RSV, PIV1, PIV2, PIV3, ADV and MPV. Each clinical specimen was also tested for the human ribonuclease P gene to measure nucleic acid integrity and to confirm sample adequacy. A qRT-PCR test result was considered positive if an exponential

fluorescence curve was produced that crossed the assigned threshold at Ct <40.0. The results of direct immunofluorescence for all of the former, Flu A/B, PIV1-3, RSV, AdV, MPV, plus blood culture followed by confirmatory PCR for *Bordetella pertussis*, performed at the hospital facilities as part of the diagnostic testing required for the patients, were also available.

DNA sequence analysis of NP1 and VP1 regions

Seventeen samples were used for sequence analysis. A region corresponding to the NP1 protein of HBoV was amplified by nested PCR as described before [6]. Amplification of the complete VP1 region was carried out using three overlapping pair primers, with 5 µl of DNA template in a total volume of 50 µl per reaction, including 0.25 mM dNTP mix, 5 U of Platinum Taq DNA polymerase (Invitrogen) per µl and the following primers at a concentration of 0.40 µM: HBoV1_2945F (5′ATTACTGGGATGATGT GTACCGT) and HBoV1_3724R (5′CCATGGAGTTGTG ACGCAGC), HBoV1_3339F (5′TGGGAAATAAAGAGA GAGCCCAA) and HBoV1_4288R (5′TGCTGTGCTTCC GTTTTGTCT), HBoV1_4138F (5′ACT TAGAACTGGTG AGAGCACTG) and HBoV1_5127R (5′CCGCTTGTCC ATTGAGGAGGA). PCR products were resolved in 2 % agarose gels stained with ethidium bromide and purified using a QIAGEN PCR cleanup kit. Sequencing reactions were performed bidirectionally using appropriate primers and cycle-sequencing kits (ABI PRISM BigDye Terminator v. 3.1; PE Applied Biosystems) and resolved in a 3700 Genetic Analyzer (Applied Biosystems). For phylogenetic analysis using the NP1 region, in addition of the seventeen local isolates (6 from 2011 and 11 from 2012) the following sequences were included: JN632487, JN632491, JX034730 (local isolates from previous years) and representative sequences available in the GenBank database (HBoV1, EF203921; HBoV1, DQ000496; HBoV1, DQ000495; HBoV1, DQ340570; HBoV1, JQ411251; HBoV2, GU048663; HBoV2, GU048662; HBoV2, GU048664; HBoV2, FJ170279; HBoV2, FJ170280; HBoV2, GQ200737; HBoV2, EU082214; HBoV2, FJ948860; HBoV3, HM132056; HBoV3, GU048665; HBoV3, FJ948861; HBoV3, EU918736; HBoV3, CQ867666; HBoV4, FJ973561; CnMV, AB158475; CnMV, AF495467; and BPV, DQ335247). Phylogenetic analysis of the VP1 region was performed using 10 local isolates from previous years (five from 2009, one from 2010 and four from 2011).

For cataloguing and storage, sequences were input into free online sequence-alignment software (ALIGN Query, GENESTREAM SEARCH network server IGH, Montpellier, France; http://xylian.igh.cnrs.fr/bin/align-guess.cgi). Phylogenetic analysis was conducted with MEGA version 5.03 software (www.megasoftware.net) using the neighbor-joining method; bootstrap values were calculated for 1000 replicates.

Clinical and epidemiological data

Clinical and epidemiological features of patients ≤14 years old, admitted at the Children's Hospital of Cordoba City, were recorded in ad-hoc forms. Medical records included demographic data, history of illness, risk factors or co-morbidity, clinical symptoms, blood laboratory results, chest X-rays radiographic findings, diagnosis, antimicrobials or other drugs prescribed, symptomatic support therapy, and evolution of illness.

Statistical analysis

Quantitative and qualitative variables were compared using Student's t-test or chi square test with a level of significance $p < 0.05$

Results

Description of study population

The average age of 843 patients (443 male and 400 female) enrolled in the study was 4.56 years old (standard deviation [SD], 11.13 yr; median age, 0.5 yr), with 606/843 (71.9%) infants ≤1 year old. The majority of the samples, 659 (77.9%) were collected during the months corresponding to fall or winter. During the same sampling period of this study, 292 asymptomatic individuals were enrolled; the age range was 10 month to 62 years old, average 15.42 years old (SD, 11.33 yr; median age, 11 yr).

Detection of HBoV in patients with ARTI

HBoV DNA was detected in 78 of 843 patients with ARTI, resulting in a general positive rate of 9.25 %. There were 24/379 (6.33 %) positive cases in 2011 and 54/464 (11.64 %) in 2012. HBoV+ samples were found throughout the complete period studied, although most HBoV+ cases, 60 out of 78 (76.9 %), occurred during late fall through winter (Fig. 1A). There was a wide age range of HBoV genome detection, including neonatal patients of one month to adult patients (23 years old). However, 60 of 78 (76.9 %) cases were infants less than 1 year old (Fig. 2). The mean age of HBoV+ cases was 1.94 years old (SD, 3.87 yr; median, 8 months). The youngest positive patient was 30 days old. Among HBoV+ patients, 46 of 78 (59 %) were male. Since there was a significant ($p < 0.01$) difference in the prevalence of

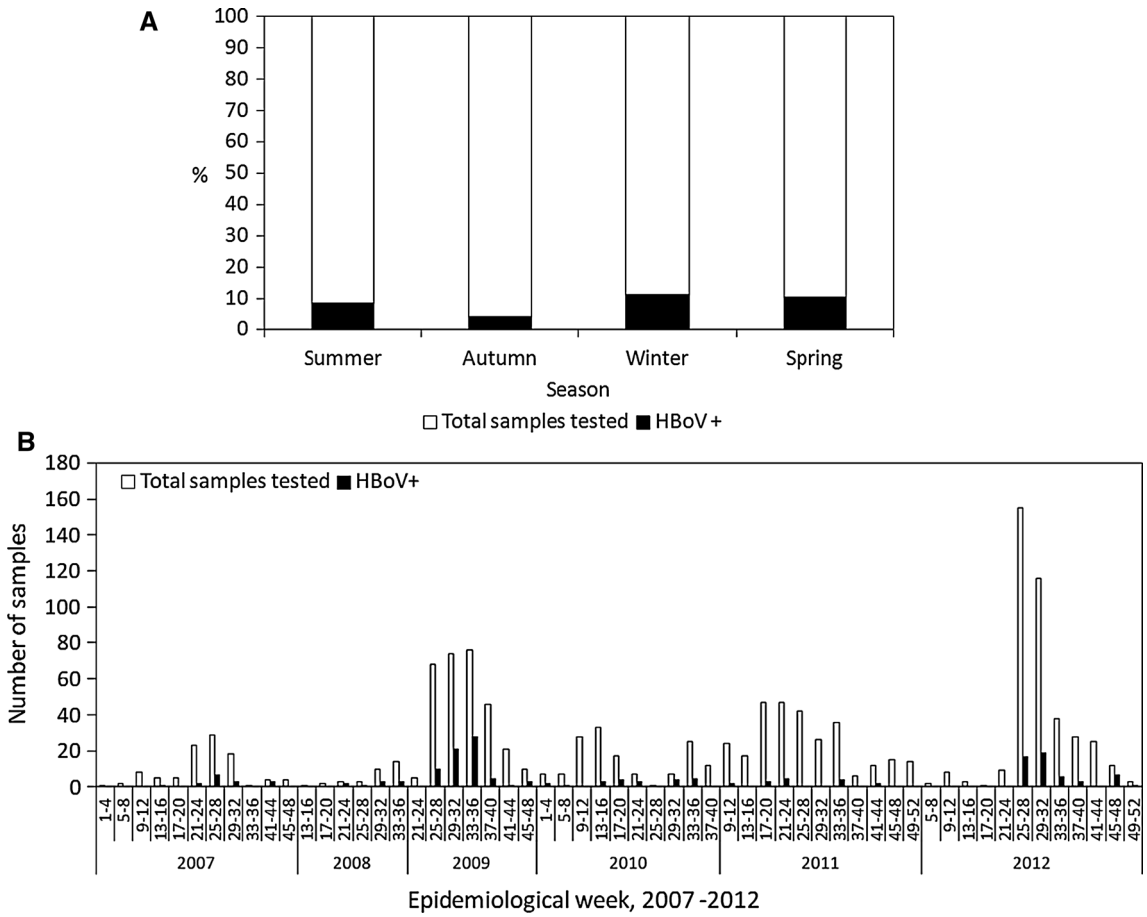

Fig. 1 **A** Epidemiological seasonal distribution of HBoV in a group of 843 patients with ARTI in Córdoba, Argentina, in 2011–2012. **B** Epidemiological weekly distribution of HBoV, 2007-2012

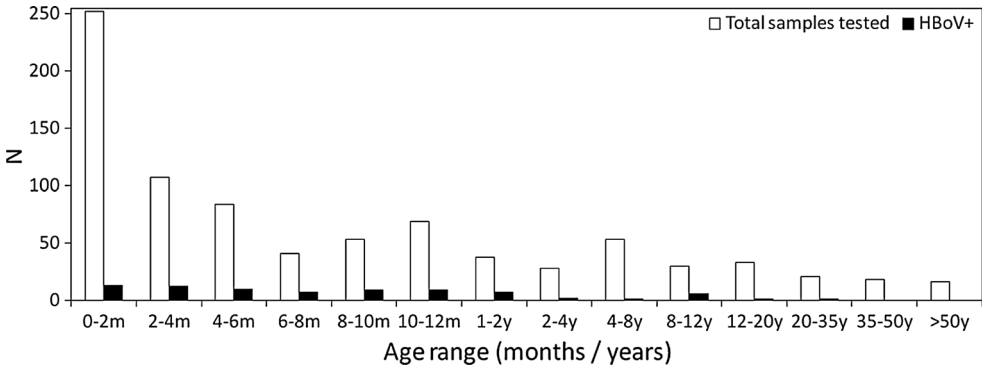

Fig. 2 Age distribution of HBoV among patients 0-70 years old with ARTI in Córdoba, Argentina, in 2011–2012

HBoV in 2011 and 2012, we pooled data of detection in pre-school-aged children with ARTI ≤5 years old for the period 2007-2012 in order to detect periodicity of circulation. As shown in Figure 1B, HBoV frequency can fluctuate widely from year to year; an epidemic peak was observed in 2009, and the present period could be interpreted as inter-epidemic. On the other hand, 11 out of 292 (3.77 %) asymptomatic individuals were HBoV+. The age of HBoV+ control participants was 9 to 12 years old (mean age, 11.18 yr; SD, 0.98 yr; median, 11 yr); all of these cases occurred in classrooms of the same school. The difference in HBoV frequency observed in patients with ARTI and asymptomatic controls was statistically significant ($p < 0.01$).

Table 1 Co-detection of other common pathogens in HBoV+ samples from children with ARTI (n = 73)

Co-pathogens	No. of positive patients (%)	
	IF or culture/PCR[1]	PCR
Simple co-infection		
RSV	23 (29.5)	41 (56.2)
Flu A	4 (5.2)	0 (0)
Flu B	0 (0)	0 (0)
PIV1	0 (0)	0 (0)
PIV2	0 (0)	0 (0)
PIV3	3 (3.8)	3 (4.1)
AdV	0 (0)	2 (2.7)
MPV	0 (0)	1 (1.3)
Bordetella pertussis[2]	2 (2.6)	0 (0)
Multiple co-infection		
RSV + AdV	0 (0)	4 (5.5)
RSV + Flu A	0 (0)	1 (13.7)
RSV + PIV 3	0 (0)	1 (13.7)
RSV + *Bordetella pertussis*	0 (0)	2 (2.7)
RSV + AdV + MPV	0 (0)	1 (13.7)
RSV + AdV + PIV3	0 (0)	1 (13.7)
TOTAL	32/73 (43.8)	57/73 (78.1)

7 out of 74 HBoV+ samples could not be screened due to an insufficient amount of template/clinical specimen

[1] Diagnostic assay performed at the hospital for common germs, including RSV, Flu, PIV and MPV respiratory viruses by IF assay and blood culture

[2] Diagnosis by blood culture followed by confirmatory PCR

Co-detection of common respiratory pathogens in HBoV+ samples

HBoV+ samples were screened by real-time PCR in order to determine the rate of co-infection with other common viral respiratory pathogens. Another viral pathogen was detected in 57 out of 73 (78.1 %) HBoV+ samples. RSV was the most frequent co-detected virus (51/57, 89.5 %). In addition to double infections, there were eight cases of triple infection and two cases with four pathogens co-detected (Table 1); RSV was implicated in all 10 cases of triple and quadruple infections. As shown in Table 1, when co-detection was estimated, taking into account the results from assays performed at the hospital diagnostic core lab (where diagnosis was based on the immunofluorescence technique for a panel of eight respiratory viruses and blood culture), the frequency of co-detection was lower (32/78, 41.02 %).

Clinical presentation of HBoV+ patients

The clinical characteristics of pediatric HBoV+ patients admitted at the Children's Hospital of Cordoba City (patients <14 years old) are listed in Table 2. Common symptoms among these children included wheezing (88.7 %), cough (78.9 %), fever (59.3 %), rhinitis (43.7 %) and leukocytosis (32.4 %); 62 patients were clinically diagnosed with lower respiratory infection, including bronchiolitis (32 patients) and pneumonia (30 patients). In addition, 22 of the HBoV+ patients exhibited comorbidities (asthma, Down's syndrome, cardiopathy, bronchopulmonary dysplasia and microcephaly), and in six patients, the diagnosis was exacerbation of asthma. On the other hand, none of the HBoV+ patients showed poor outcome. HBoV+ patients with and without comorbidity were compared, resulting in a significant difference observed between the two groups with respect to the age of the patients (mean age with comorbidity 4.5 ± 4.72 years old; without comorbidity, 0.69 ± 0.99 years old).

To better understand the pathogenicity of HBoV, we analyzed nine HBoV+ patients with no evident coinfection. These cases occurred throughout the year (late summer to spring; range of epidemiological weeks: 11-48). The age of patients ranged from 1 month to 9 years old (mean, 1.44 yr; SD, 2.85; median, 0.5 months). Five of them were diagnosed with pneumonia, all required hospitalization during an average period of 7.88 ± 5.46 days, and six required oxygen therapy. All of the patients recovered without further complications. The most frequent symptoms among these patients with HBoV mono-infection were wheezing, cough and fever (Table 3). More analysis was carried out by comparing clinical and epidemiological data in mono- and coinfection cases. No significant differences were observed with respect to the features compared (Table 3).

Quantitative analysis of HBoV DNA in respiratory samples

The HBoV DNA load was assessed by a relative quantification technique in 30 positive samples from symptomatic individuals and in eight positive samples from asymptomatic controls. Among patients with ARTI, 13 had samples with high viral load and 17 had low viral load. All samples from asymptomatic individuals had a low viral load. The differences in viral load between these two group (controls and patients with ARTI) was statistically significant ($p < 0.01$). Clinical and epidemiological data of patients grouped according to viral load (high or low) are shown in Table 4. In the comparison of features between these two groups, no significant differences were observed.

Sequences and phylogenetic analysis

All HBoV isolates sequenced in this study clustered with HBoV1, and their sequences were nearly 100 % identical

Table 2 Clinical and epidemiological characteristics of <14 year-old HBoV+ patients with and without comorbidity admitted at the Children's Hospital of Cordoba City

	Total	No comorbidity	Comorbidity	p-value
Number of patients	71	49	22	-
EPIDEMIOLOGICAL FEATURES				
Age (years)	1.72 ±3 09	0.69 ± 0.99	4.5± 4.72	5.4 E-06*
Male/female ratio	10/12 (0.83)	31/18 (1.72)	11.13	0.16
Co-detection	53/67 (85.1)	37/45 (82.2)	16/22 (72.7)	0.37
Environmental pollution	22 (31)	18 (36.7)	4 (18.2)	0.12
Rural habitat	2 (2.8)	0 (0)	2 (9.1)	0.03*
Cohabitant with ARTI	4 (5.6)	4 (8.2)	0 (0)	0.19
DIAGNOSIS				
Asthma exacerbation	6 (8.5)	0 (0)	6 (27.3)	0.001*
Bronchiolitis	33 (46.5)	29 (59.2)	4 (18.2)	0.001*
Pneumonia	30 (42.3)	20 (40.8)	10 (45.5)	0.71
Rhinitis	1 (1.4)	0 (0)	1 (4.6)	0.04*
Laryngitis	1 (1.4)	0 (0)	1 (4.6)	0.04*
COMORBIDITY				
Asthma	6 (8.5)	-	6	-
Microcephaly	1 (1.4)	-	1	-
Bronchopulmonary dysplasia	2 (2.8)	-	2	-
Heart disease	4 (5.6)	-	4	-
Down syndrome	4 (5.6)	-	4	-
Other	5 (7.1)	-	5	-
CLINICAL SIGNS				
Fever (temperature ≥38 °C)	42 (59.2)	29 (59.1)	13 (59)	0.99
Cough	56 (78.9)	35 (71.4)	21 (95.5)	0.02*
Emesis	12 (16.9)	8 (16.3)	4 (18.2)	0.84
Apnea	4 (5.6)	4 (8.1)	0 (0)	0.16
Cyanosis	6 (8.5)	5 (10.2)	1 (4.6)	0.42
Rhinitis	31 (43.7)	22 (44.8)	9 (40.9)	0.75
Wheezing	63 (88.7)	44 (89.8)	19 (86.4)	0.67
Diarrhea	5 (7.1)	5 (10.2)	0 (0)	0.12
Poor outcome	11 (15.5)	7 (14.3)	4 (18.2)	0.67
Days of hospitalization	7.11 ± 4.87	6.83 ± 4.42	7.72 ± 5.79	0.48
Days of oxygen therapy	4.18	4.4	3.59	0.34
Prodromal period	3.43 ± 2.64	3.53 ± 2.87	5.79 ± 2.04	0.64
Erythrosedimentation rate	24.7	22.22	29.9	0.04*
Leukocytosis	20 (28.2)	13 (2)	7 (31.8)	0.64

* Significant statistical differences between HBoV+ patients with and without comorbidity

in the NP1 gene, showing that they are highly conserved. Only three G-A transitions were observed. The average genetic distance among the 17 local NP1 sequences was 0.001. Phylogenetic analysis of this ORF in HBoV strains from Argentina did not reveal any genotypic differences when compared to strains from Sweden and other countries (average distance, 0.001; Fig. 3A). The genetic distance among local isolates considering the complete VP1 region was higher (0.005 among local isolates – see Fig. 3B – and 0.006 among local and reference sequences DQ000496, DQ000495, EF203921, DQ340570), as could be expected for a structural protein exposed to the pressure of the immune response of the host.

Nucleotide sequence accession numbers

The sequences of PCR products of HBoV1 NP1 and VP1 were deposited in the GenBank database under accession numbers JX034731 to JX034736, KC878500 to KC878510, and KC544960 to KC544969 (see Fig. 3).

Discussion

Since human bocavirus was first discovered in 2005 [1] it has been associated with acute upper and lower respiratory disease. Most of the studies have focused on children less

Table 3 Clinical and epidemiological characteristics in single HBoV infection and in coinfection with other respiratory pathogen in patients <14 years old admitted to the Children's Hospital of Cordoba City

	Single HBoV infection[1]	Coinfection	p-value
EPIDEMIOLOGICAL FEATURES			
HBoV frequency	9/67	58/67	
AGE of patients (years)	1.44 ± 2.84	1.91 ± 3.3	0.68
HBoV+ male /female rate	4/5 (0.8)	35/23 (1.52)	0.36
Environmental pollution	1 (11.1)	20 (34.5)	0.15
Rural habitat	1 (11.1)	1 (1.7)	0.12
Contact with a cohabitant with ARTI	1 (11.1)	2 (3.4)	0.30
DIAGNOSIS IN HBoV+ PATIENTS			
Asthma exacerbation	1 (11.1)	5 (8.62)	0.8
Bronchiolitis	2 (22.2)	28 (48.3)	0.21
Pneumonia	5 (55.5)	24 (41.4)	0.42
Rhinitis	1 (11.1)	0 (0)	0.01
Laryngitis	0 (0)	1 (1.7)	0.69
Comorbidity	4 (44.4)	15 (25.9)	0.24
CLINICAL SIGNS IN HBoV+ PATIENTS			
Malnutrition	2 (22.2)	12 (20.7)	0.91
Fever (temperature ≥38 °C)	4 (44.4)	33 (56.9)	0.48
Cough	5 (55.5)	48 (82.8)	0.06
Emesis	2 (22.2)	10 (17.2)	0.71
Apnea	0 (0)	4 (6.9)	0.41
Cyanosis	0 (0)	6 (10.3)	0.31
Rhinitis	1 (11.1)	30 (51.7)	0.02
Wheezing	8 (88.8)	51 (87.9)	0.93
Diarrhea	1 (11.1)	4 (6.9)	0.65
Poor outcome	2 (22.2)	9 (15.5)	0.61
Days of hospitalization	7.88 ± 5,46	7.25 ± 4.86	0.72
Prodrome	4.7 ± 4.21	3.2 ± 2.3	0.11
Days of oxygen required	2.78 ± 2.38	4.56 ± 3.62	0.15
Erythrosedimentation rate	26.44	24.67	0.74
Leukocytosis	3 (33.3)	20 (34.5)	0.94

[1] Negative for IFI and PCR

Table 4 Clinical and epidemiological characteristics of patient with high or low HBoV viral load

	Low viral load	High viral load	p-value
HBOV-EPIDEMIOLOGICAL FEATURES			
HBoV+	17	13	
Age (years)	3.46 ± 4.84	1.88 ± 2.46	0.2
HBoV+ male /female	0.72	1.6	0.2
CO-DETECTION	13 (76.5)	9 (69.2)	0.65
Environmental pollution	4 (23.5)	4 (30.8)	0.67
Rural habitat	0 (0)	2 (15.4)	0.94
Contact with a co-habitant with ARTI	1 (5.9)	1 (7.7)	0.84
DIAGNOSIS OF HBoV+ PATIENTS			
Asthma exacerbation	4 (23.5)	2 (15.4)	0.58
Bronchiolitis	6 (35.3)	6 (46.2	0.54
Pneumonia	7 (41.1)	5 (38.5)	0.88
Rhinitis	0 (0)	0 (0)	Nc
Laryngitis	0 (0)	0 (0)	Nc
CLINICAL SIGNS OF HBoV+ PATIENTS			
Comorbidity	8 (47.1)	4 (30.8)	0.36
Malnutrition	2 (11.8)	2 (15.4)	0.77
Fever (Temperature ≥38 °C)	9 (52.9)	9 (69.2)	0.13
Cough	14 (82.4)	13 (100)	0.11
Emesis	3 (17.6)	1 (7.7)	0.42
Apnea	0 (0)	0 (0)	Nc
Cyanosis	0 (0)	0 (0)	Nc
Rhinitis	9 (52.9)	6 (46.2)	0.09
Wheezing	15 (88.2)	12 (92.3)	0.71
Diarrhea	1 (5.9)	1 (7.7)	0.84
Poor outcome	2 (11.8)	2 (15.4)	0.77
Days hospitalized	5.94 ± 4.53	6.69 ± 4.62	0.65
Oxygen required	3.26 ± 2.15	5.15 ± 4.66	0.17
Days of prodrome	3.06 ± 2.4	3.07 ± 2.7	0.98
Erythrosedimentation rate	28.33	28.91	0.91
Leukocytosis	5 (29.4)	5 (38.5)	0.6

than 5 years old [6, 24–28], but the virus has also been found in adult patients [4, 7]. Thus, our purpose was to describe HBoV circulation in the general population and the associated clinical and epidemiological aspects of infection. We searched for the virus in respiratory specimens of individuals of all ages with and without ARTI, genotyped isolates, estimated viral loads, and analyzed clinical presentation of positive patients, with and without coinfection and comorbidity. Our results suggest that HBoV1 is a cause of respiratory disease.

In the present study, the general positive rate of HBoV in patients with ARTIs was 9.25 % (78/843) and 6.33 % (24/379) in 2011 and 11.64 % (54/464) in 2012. The yearly frequency, considered together with results from similar datasets from previous years [4, 6] indicates annual variations in the circulation of HBoV respiratory infection (Fig. 1B).

Every year, HBoV cases are more prevalent during the winter season (epidemiological weeks 25 to 36, Fig. 1A), in agreement with most studies [14, 26], even when the prevalence has been reported to be high during warm seasons [16, 29].

Primary infection with HBoV seems to occur early in life; infants between 6 and 24 months of age have been reported to be the most frequently affected group [30, 31]. This has led to the suggestion that infants younger than

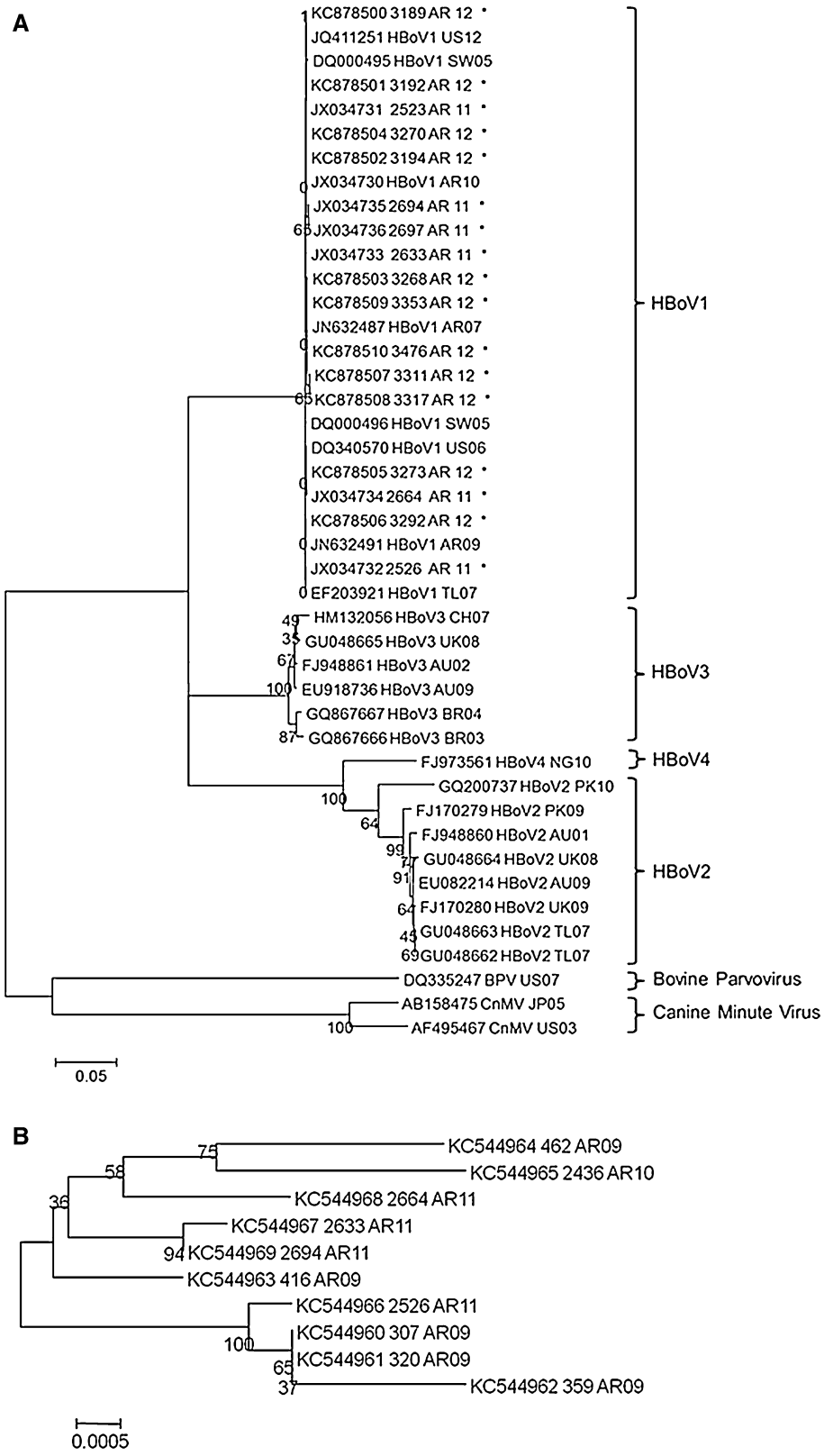

Fig. 3 Phylogenetic trees based on HBoV NP1 and VP1 sequences, with 1,000 bootstrap replicates, using the neighbour-joining method. **A** Phylogenetic relationships among HBoV1, HBoV2, HBoV3, HBoV4, CnMV, BPV and 17 local HBoV sequences (*) amplified after nucleic acid extraction from NPA of pediatric patients with ARTI. The phylogenetic analysis was done using the region between nucleotides 2321 and 3056 (NP1) of the HBoV1 genome (accession no. DQ000496). **B** Phylogenetic relationships among 10 local sequences. The phylogenetic analysis was done using the region between nucleotides 3056 and 5071 (VP1) of the HBoV1 genome (accession no. DQ000496). Accession numbers of the local strains are shown in the figure

6 month are protected by maternal antibodies [32, 33]. However, we found that 44.9 % of HBoV-positive cases occur in patients less than 6 months old and 76.9 % in patients less than 1 year old (Fig. 2). Furthermore, we detected HBoV infection in seven newborns. This shows a very early incidence of infection and at the same time

suggests that maternal antibodies are lost quickly or are not completely able to provide protection against HBoV infection. This would be in line with the re-infection hypothesis proposed by Meriluoto et al. [18]. Alternatively, vertical transmission of HBoV infection could be considered.

In contrast to some authors who did not find HBoV in asymptomatic individuals [34, 35], we observed a 3.77 % positive rate in the control group. All of the positive samples were obtained from two classes in the same school, suggesting a small outbreak in the school. This also shows that infection can occur at relatively high frequency in school-aged children.

The main clinical features in HBoV-positive patients (Table 2) were wheezing (88.7 %), cough (78.9 %), fever (59.3 %), rhinitis (43.7 %) and leukocytosis (32.4 %), and 87 % of HBoV-infected patients were clinically diagnosed with lower respiratory infection (bronchiolitis, pneumonia). Nonetheless, further severe complications or deaths were not observed in these patients, in agreement with previous studies [36–39]. It is noteworthy that 30 % of HBoV-positive patients exhibited comorbidities, and a significant difference was observed in the age of patients with and without co-morbidity. According to our results, while HBoV infection is common during the first year of life in previously healthy children, those with comorbidities are susceptible to infection at even older ages.

Similar to previous studies, a high rate of coinfection with other pathogens was common among HBoV-infected patients [8, 36, 40, 41]. In the present study, the majority of the cases of coinfection (78.1 %) occurred with RSV (89.5 %, Table 1), which has been reported in a number of other papers as well [7, 9, 12], even though variations in the seasonal peaks occur for each virus every year. Thus, it is worth investigating whether any relationship or interaction exists between these two viruses, other than co-circulation.

No significant differences were observed with respect to clinical and epidemiological aspects in patients with HBoV single infection compared to patients with coinfection (Table 3). This indicates that coinfection does not increase the duration or severity of illness, which in turn strongly suggests the pathogenic potential of HBoV in young children with ARTI. In other words, the virus can be pathogenic by itself, as other respiratory viruses are, in single or multiple infection events. Furthermore, we investigated the correlation between viral load and severity of disease, but in contrast to the findings of other authors [14, 15], we did not find an association between high HBoV load and disease (Table 4). We did, however, detect only low viral loads in asymptomatic individuals ($p < 0.05$), and other authors found similar results [7]. Based on the analysis of clinical presentation with respect to viral load in patients with ARTI, high HBoV load could have a significant influence on the clinical presentation of the infection, but considering that patients with ARTI can actually have a high or low HBoV load, it can be proposed that the clinical presentation of infection occurs depending on both viral load and the particular interaction between the HBoV and the host.

Even when no distinctive clinical signs or symptoms of HBoV infection could be identified, similar to other studies [6, 14, 16, 42, 43], it is worth noting that among hospitalized pediatric patients, HBoV infection is mostly associated with a diagnosis of pneumonia and bronchiolitis, two major diseases of infancy with a high impact on the health system.

Complete NP1 and VP1 sequence of local isolates were obtained. Genetic analysis showed a high degree of sequence identity among local strains (the genetic distance was 0.001 in the NP1 segment, Fig. 3A, and 0.005 for VP1, Fig. 3B) and when compared to other strains around the world (the genetic distance was 0.001 for NP1, Fig. 3A, and 0.006 for VP1). The phylogenetic tree shows that all our strains belong to the cluster of HBoV1 and are closely related to the original virus reported by Allander et al. [1]. The genetic distance was slightly higher for the coding region of VP1 than for NP1. However, it remains to be elucidated if the genetic distance in the structural protein fragment is enough to be the underlying cause of re-infections, i.e., the reason why a high rate of infection is consistently detected in children despite the accumulation of immune individuals [4, 6].

In conclusion, our results support that HBoV1 is a causative agent of acute respiratory disease, mainly associated with bronchiolitis and pneumonia in children. Host features or virus-host interactions – yet to be identified but possibly involving persistent infection [44, 45] – may influence the outcome of infection. High viral load could be proposed as a factor linked to disease, since one major difference between patients with ARTI and asymptomatic individuals is the consistently low viral load in healthy HBoV-positive individuals.

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Conflict of interest All authors declare that they have no conflict of interest.

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