The human bocavirus role in acute respiratory tract infections of pediatric patients as defined by viral load quantification

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SUMMARY

The major objective of this study was to investigate the pathogenic role of human bocavirus (hBoV) in patients hospitalized with acute respiratory tract infection (ARTI). Overall, 685 respiratory samples from 426 patients were examined by PCR for human bocavirus, as well as for other known human respiratory viruses. HBoV was quantified by PCR. Forty/283 (14.1%) pediatric patients, and 2/143 (1.4%) adult patients were found to harbor hBoV for a total of 45 episodes (16 detected as single infection, and 29 as coinfection) of hBoV-associated respiratory infection. HBoV DNA quantification revealed the presence of an NPA viral load >$1.0x10^5$ DNA copies/ml in respiratory secretions from 17/40 (42.5%) children and 0/2 adults. Below this cut-off, hBoV appeared to be an innocent bystander or a persistent virus. Although hBoV may be frequently detected in children with upper or lower ARTI, in less than 50% young patients it appears to be potentially pathogenic.

KEY WORDS: Human bocavirus, Acute respiratory tract infection, Viral load, Nasopharyngeal aspirate

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INTRODUCTION

In 2005, the group of respiratory viruses pathogenic for man increased by a new member: the human bocavirus (hBoV). The name Bocavirus was derived from the two initial letters of bovine (Bo) parvovirus and the minute virus of canines (ca), the two already known members of the same genus infecting the relevant animal species. Following the initial report (Allander *et al.*, 2005), hBoV has been associated with both upper and lower acute respiratory tract infections (ARTI) of infants and young children, but also of adults.

HBoV has been reported to circulate with a frequency of 1.5% to 11.3% or more in patients with acute respiratory illness with an average frequency of 5.0-5.5% in the majority of the studies (Allander *et al.*, 2005; Kesebir *et al.*, 2006; Manning *et al.*, 2006; Arnold *et al.*, 2006; Bastien *et al.*, 2006a; Choi *et al.*, 2006; Foulongne *et al.*, 2006; Ma *et al.*, 2006; Sloots *et al.*, 2006; Maggi *et al.*, 2007). This makes the circulation frequency of these viruses the highest after human respiratory syncytial virus (hRSV) and rhinoviruses (McIntosh, 2006). Infants and young children are the individuals mostly affected by hBoV infections, with a few exceptions (Bastien *et al.*, 2006a). Winter-spring is the season generally showing the highest hBoV circulation rate with some reports describing cases of hBoV infection in late fall or

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early summer or even throughout the year (Bastien *et al*., 2006a). However, from the start the most intriguing question related to hBoV infections was the pathogenicity of this virus. Is hBoV always or sometimes pathogenic or is it just a bystander virus? (Weissbrich *et al*., 2006; Lu *et al*., 2006). This issue is made even more difficult to investigate by the elevated number of hBoV coinfections reported in all studies, an incidence never reported for any other respiratory virus (Allander *et al*., 2005; Sloots *et al*., 2006; Chung *et al*., 2006; Kaplan *et al*., 2006; Fry *et al*., 2007). The frequency of coinfections ranged from 15% to more than 70%, and in some cases hBoV was associated with two different respiratory viruses. In this respect, significant progress towards the clarification of this issue has recently been made thanks to the contribution of some studies examining a substantial number of control individuals in the absence of respiratory symptoms in parallel with patients with respiratory disease (Kesebir *et al*., 2006; Manning *et al*., 2006; Maggi *et al*., 2007; Fry *et al*., 2007). These studies failed to detect hBoV or detected hBoV very infrequently in this control patient population.

In the present study, hBoV was sought in 426 patients admitted to hospital in the winter-spring season 2005-2006 for an episode of acute respiratory tract infection (ARTI) and was detected in 42 patients (40 infants and young children, and 2 adults) either as a single infection (n=16 episodes) or in association with other viruses (n=29 coinfection episodes). In each patient, we attempted to identify the hBoV etiologic or bystander role by means of DNA quantification in respiratory secretions. Results seem to indicate a discriminatory potential of virus quantification. However, in some patients a high virus replication rate was not reported to be associated with severe clinical symptoms.

MATERIALS AND METHODS

Patient population and test samples

The study involved a total of 426 patients, including 283 infants and young children, and 143 adults. On the whole, 686 respiratory samples, i.e. 552 (80.5%) nasopharyngeal aspirates (NPAs), and 134 (19.5%) bronchoalveolar lavage (BAL) samples, were examined. The total number of patients with respiratory episodes was 268 (62.9%), while the total number of respiratory episodes was 366, including both episodes of single infection (n=278, 75.9%) and episodes of coinfection (n=88, 24.1%). Respiratory specimens were collected from 1 November 2005 through 31 May 2006 by trained personnel using a standardized procedure (DeVincenzo, 2004), and originated from patients seen in the emergency department, inpatient wards, and intensive care units as well as from patients at control visits after hospital discharge. These specimens were tested for influenzaviruses A and B (Flu A and B), human parainfluenzaviruses 1-4 (hPIV), human respiratory syncytial virus (hRSV), human adenoviruses (hAdV), human coronavirus (hCoV) 229E and HKU1, and human metapneumoviruses (hMPV) by monoclonal antibodies (MAbs) and direct fluorescent antibody staining (DFA) prior to and after inoculation onto cell cultures. On the other hand, hCoV-NL63 and hCoV-OC43, hMPV, and human rhinoviruses (hRhV) were detected by RT-PCR (Sarasini *et al*., 2006; Gerna *et al*., 2007). In addition, semiquantification of coinfecting viruses was achieved by the endpoint dilution method.

HBoV detection and quantification by qPCR

In detail, hBoV was detected by PCR as follows. Following extraction of nucleic acids from 200 µl of clinical samples (NPA or BAL) by means of automatic extraction kit Nuclisens® easy MAG™ (BioMérieux, Lyon, France), 10 µl of the elution volume (50 µl) underwent DNA amplification using a primer pair targeting the NS1 gene, as reported elsewhere (Sloots *et al*., 2006). PCR amplification cycles were performed at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s, 56°C for 15 s, and 72°C for 20 s + 5 s/cycle, and then by a final extension cycle at 72°C for 10 min. The reaction mixture (50 µl/sample) consisted of 1.5 mM $MgCl_2$ PCR Buffer (Applied BioSystem, Foster City, CA), dNTP (250 µmol each), primer pair (500 nmol each), 100 copies of internal DNA control, 1.5 U of AmpliTaq Gold® (Applied BioSystem), H_2O to final volume of 40 µl, and 10 µl of sample extract. The amplification product (290 nt) was electrophoresed in 3% agarose gel, and positive samples were quantified by qPCR using a plasmid containing the PCR product serially diluted in the range of 10^5 to 10^0 input copies to construct external reference standards.

The internal DNA control consisted of a plasmid carrying the recognition sites of hBoV-specific primers at the ends of a DNA heterologous sequence with a nucleotide size (~400 nt) different from that of the target hBoV sequence. A fixed amount of internal control (100 copies) was added to external standards as well as to test samples and co-amplified using the same primer set. A standard curve was constructed from densitometric values of ratios of single external standards and internal control, from which values of viral load of test samples were interpolated. The sensitivity of the assay was at least 10 DNA copies. Results were adjusted to provide DNA copy number/ml NPA or BAL. The mean intraassay coefficient of variation (CV) was 8.6%, while the interassay CV was 11.1%. The recent development in our laboratory of a real-time PCR assay for hBoV DNA detection targeting VP1/VP2 genes according to Lu *et al.* (2006) showed an interassay CV of 6.0%, while the amount of hBoV DNA detected by real-time PCR was not significantly different (p=0.88) from that detected by qPCR.

DNA sequencing and phylogenetic analysis

Following amplification of a region of VP1/VP2 genes by a primer pair reported elsewhere (Kesebir *et al.*, 2006), and following amplicon purification, nucleotide sequencing was performed on both amplicon strands by applying the ABI PRISM™ Big Dye Terminator kit (Applied Biosystems). Sequencing was performed using an Applied Biosystems 3100 Genetic Analyzer. Viral sequences were aligned with the ClustalW program, version 1.7, while the Mega 3.1 program was used to construct phylogenetic trees with nucleotide sequences by means of the neighbour-joining method from the same distance matrices. Bootstrap support was determined by 1,000 sequence resamplings.

Statistical analysis

Using $1x10^5$ DNA copies/ml as a threshold value, comparison of median values of the two groups of patients with higher and lower viral load levels was done by using the Mann-Whitney U test. In addition, the Fisher's exact test was used to determine differences in frequency.

RESULTS

General incidence of respiratory virus infections in the winter-spring season 2005-2006

Of the 366 episodes of respiratory virus detection involving 268/426 (62.9%) patients, 278 (75.9%) were episodes of single infection, and 88 (24.1%) episodes of co-infection or co-detection of two or three respiratory viruses in the same sample of respiratory secretions.

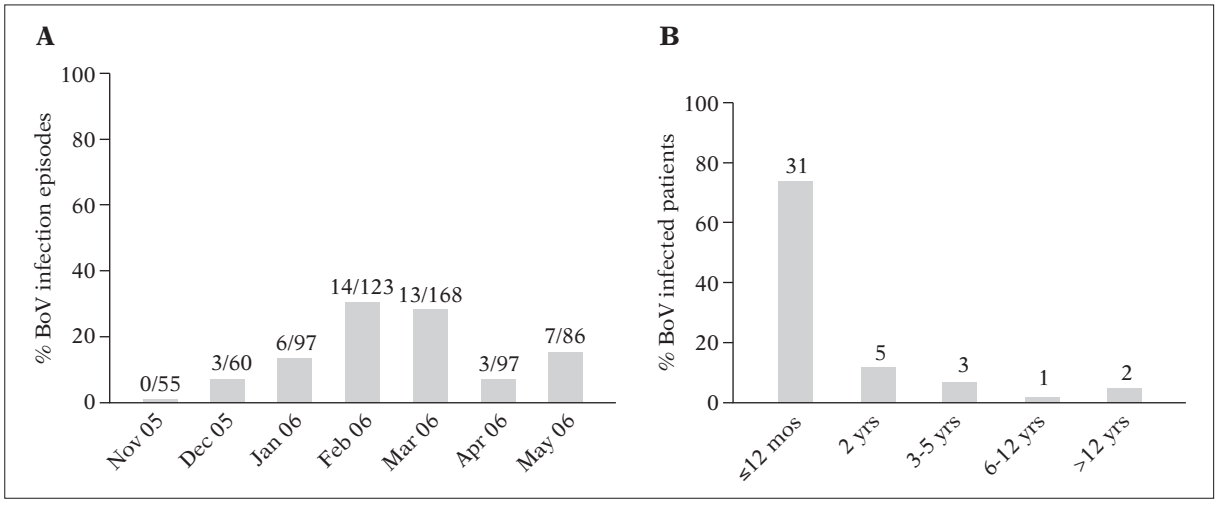

FIGURE 1 - *(A) Relative monthly distribution of episodes of hBoV infection during the winter-spring season 2005-2006. At the top of each column the ratio of positive to total number of samples examined is reported. (B). Age distribution of patients with hBoV infection during the same season. At the top of each column the total number of hBoV-positive patients is reported.*

TABLE 1 - *Human bocavirus (hBoV) nosocomial transmission.*

Patient #	*Age*	*Days after admission*	*Virus detected at admission*	*Viral load quantification (copies/ml NPA)*		*Clinical symptoms/ syndrome*	*Inferred etiologic involvement*
				hBoV	*Other viruses*		
3	2 mos	0	hRSV	neg	hRSV: $1.8x10^7$	bronchiolitis	hRSV-A
		3		$1.5x10^6$	hRSV: $4.6x10^5$	fever	hRSV-A+hBoV
10	1 yr	0	hMPV+hCoV-OC43	neg	hRhV: $<1.0x10^3$	otalgia	hMPV+hCoV-OC43
		9		$1.7x10^6$	hRhV: $2.5x10^6$	rhinorrhea	hRhV+hBoV
11	8 mos	0	hPIV	neg	hRhV: $<1.0x10^3$	bronchiolitis	hPIV
		5		$2.9x10^5$	hRhV: $2.5x10^7$	none	hRhV+hBoV
18-1	3 yrs	0	hPIV	neg		pneumonia	hPIV (+ *M. pneumoniae*)
		7		$2.9x10^4$		resolving pneumonia	hBoV

hRSV, human respiratory syncytial virus; hMPV, human metapneumovirus; hCoV, human coronavirus; hRhV, human rhinovirus; hPIV, human parainfluenza-virus.

TABLE 2 - *Human bocavirus (hBoV) acquisition in a health care setting.*

Patient #	*Age*	*Days after admission*	*Virus(es) detected at admission*	*Viral load quantification (copies/ml NPA)*		*Clinical symptoms/ syndromes*	*Inferred etiologic agent(s)*[a]
				hBoV	*Other viruses*		
2	6 mos	0	hBoV, hRSV, hRhV	$1.7x10^6$	hRSV: $9.4x10^6$ hRhV: $2.5x10^7$	bronchiolitis	hRSV-A+hBoV+hRhV
		11		neg	hRSV: $3.5x10^4$ hRhV: $<1.0x10^3$		
23-6	1 mo	0	hRSV-B, hBoV	$5.0x10^2$	hRSV: $3.7x10^5$	pneumonia	hRSV-B+[hBoV]
		8		neg	hRSV: $2.2x10^5$		
28-11	7 mos	0	hRSV-A, hBoV	pos ($<1.0x10^3$)	hRSV: $1.4x10^6$	bronchiolitis	hRSV-A+[hBoV]
		7		neg	hRSV: $1.0x10^5$		
30-13	5 yrs	0	hPIV, hBoV	$4.2x10^4$	hPIV: $2.5x10^6$	pneumonia	hPIV+[hBoV]
		6		neg	hPIV: pos ($<1.0x10^3$)		
37-20	1 yr	0	hAdV, hRhV, hBoV	$3.4x10^3$	hRhV: $2.5x10^3$ hAdV: $2.5x10^4$	otitis, rash	[hAdV]+[hRhV] +[hBoV]
		10		neg	hRhV: neg hAdV: neg		
40-23	1 mo	0	hRSV-A, hBoV	pos ($<1.0x10^3$)	hRSV: $7.9x10^5$	bronchiolitis	hRSV-A+[hBoV]
		6		neg	hRSV: $3.6x10^3$		

hAdV, human adenovirus. For other abbreviations, see Table 1. [a]Brackets indicate possible bystander virus(es); other viruses indicate potential etiologic agent(s).

Respiratory viruses involved in episodes of single and multiple infection, respectively, were as follows: hRhV, 71+49 =120 (32.8%); hRSV, 64+34 =98 (26.8%); hCoV, 28+30 =58 (15.8%); hBoV, 16+29 =45 (12.3%); hCMV, 28+13 =41 (11.2%); hPIV, 23+17 =40 (10.9%); hAdV, 17+12=29 (8.1%); hMPV, 19+7=26 (7.1%); influenzavirus A, 6+2 =8 (2.2%); influenzavirus B, 6+1 =7 (1.9%).

Incidence of hBoV infections

The incidence of hBoV infections involved 42/426 (9.9%) patients. As mentioned above, the number of single episodes of hBoV infection was 16/45 (35.6%), and the number of hBoV coinfections was 29/45 (64.4%). The most represented co-infection or co-detection episodes were as follows: hBoV + hRSV (n=10), hBoV + hRhV (n=6), and hBoV + hRSV + hRhV (n=3). Generally, throughout the season patients had a single episode of hBoV single infection or coinfection. However, in three patients two subsequent episodes of hBoV infection occurred.

Monthly and age distribution of hBoV infections

As reported in Figure 1A, episodes of hBoV infection occurred predominantly in February (n=14) and March (n=13), whereas no case was observed in November 2005 and June 2006. As for the age distribution, 40/283 (14.1%) young patients were less than 12 years, and as many as 36 of these (90.0%) were within the first two years of age, whereas only 2/143 (1,4%) adults ($p<0.001$) were aged more than 12 years (Figure 1B).

Nosocomial transmission

As shown in Table 1, in at least four patients (#3, #10, #11, and #18-1) hBoV was not detected upon admission to hospital, but was found in respiratory secretions during the stay in the hospital or upon discharge, i.e. a median time of 6 days (range 3-9 days) after admission. The lack of virus detection upon admission together with the high viral load found in the following days strongly suggests that hBoV infection was acquired inside the hospital.

Duration of hBoV shedding

The median duration of hBoV excretion in the six patients reported in Table 2 was 7.5 days (range 6-11 days). The duration of viral shedding did not seem to change according to the level of viral load.

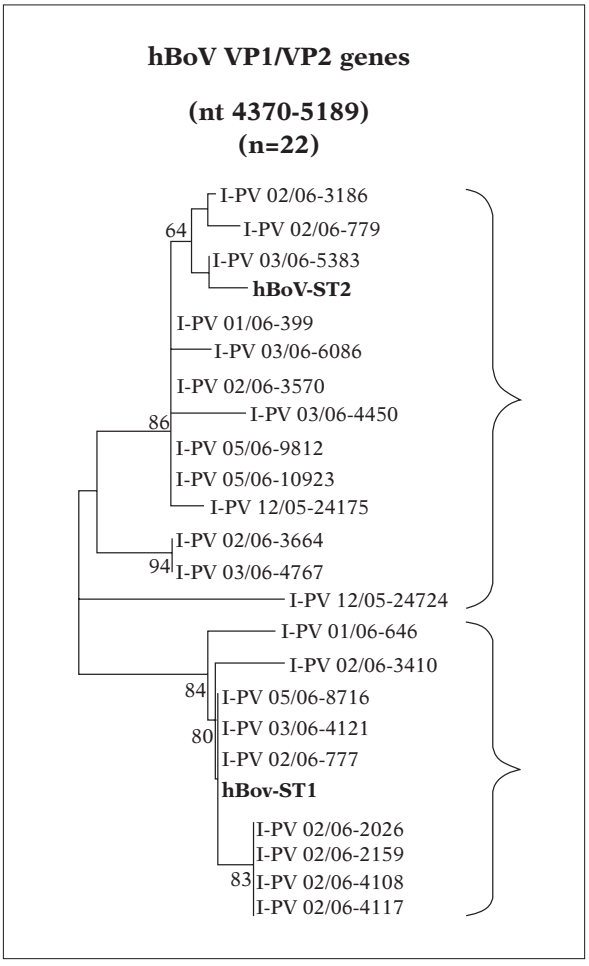

FIGURE 2 - *Phylogenetic analysis of 22 hBoV strains identified in Pavia, Italy, during the winter-spring season 2005-2006, and sequenced in a fragment of VP1/VP2 gene (nt 4370-5189). Reference strains ST1 and ST2 [1] are reported.*

Phylogenetic analysis

Phylogenetic analysis of VP1/VP2 gene fragment (nt 4370-5189) identified two distinct genotypes (Figure 2).

Out of 22 strains examined, 13 belonged to the same genotype as the ST2 reference strain, while the remaining 9 strains belonged to the same genotype as the ST1 reference strain.

Deduced role of hBoV infection in patients with high hBoV load

Table 3 reports the viral loads and clinical symptoms relevant to 17 infants and young children aged less than 26 months at time of respiratory

sample collection. In all 17 patients, hBoV load was greater than $1.0x10^5$ viral DNA copies/ml NPA, ranging from $2.2x10^5$ to $4.5x10^9$ with a median of $3.3x10^6$ copies/ml. Based on viral loads, the 17 patients could be stratified into two groups. The first group included seven patients shedding, in addition to hBoV at high viral load, one (pts #3, #7, #10, #11, #13, and #15) or two (pt #2) coinfecting virus(es) at high viral load(s). These coinfecting viruses could be considered responsible

TABLE 3 - *Infants and young children with high hBoV load in respiratory secretions at time of sample collection.*

Patient #	*Age*	*Time of hBoV detection*[a]	*Viral load quantification (copies/ml NPA)*		*Clinical symptoms/syndromes*[c]	*Potential additional etiologic agent*[d]
			hBoV	*Other viruses*[b]		
1	17 mos	C	$1.3x10^6$		rhinorrhea	none
2	7 mos	A	$1.7x10^6$	hRSV: $9.4x10^6$ hRhV: $2.5x10^7$	bronchiolitis	hRSV+hRhV
3	2 mos	S	$1.5x10^6$	hRSV: $4.6x10^5$	fever	hRSV
4	15 mos	A	$9.9x10^8$	hRhV: $2.5x10^4$	fever, cough, sore throat, wheezing	[hRhV]
5	11 mos	A	$2.5x10^9$	hRSV: $2.5x10^3$ hRSV: $<1.0x10^3$	fever, cough, wheezing	[hRSV+hRhV]
6	17 mos	A	$2.1x10^7$		pneumonia*	none
7	26 mos	A	$7.1x10^7$	hCoV: $2.5x10^5$	pneumonia*	hCoV-NL63
8	15 mos	A	$3.3x10^6$		fever, cough, otalgia, rhinorrhea, diarrhea, rash	none
9	10 mos	C	$2.5x10^7$		rhinorrhea	none
10	21 mos	A	$1.7x10^6$	hRSV: $<1.0x10^3$ hRhV: $2.5x10^6$	rhinorrhea	[hRSV]+hRhV
11	9 mos	D	$2.9x10^5$	hRhV: $2.5x10^7$	rhinorrhea	hRhV
12	6 mos	A	$2.6x10^7$		interstitial pneumonia*	none
13	5 mos	D	$2.2x10^5$	hRhV: $2.5x10^7$	bronchiolitis	hRhV
14	2 mos	S	$5.3x10^6$	hRSV: $8.5x10^3$	bronchiolitis	[hRSV]
15	2 mos	D	$4.4x10^5$	hRhV: $2.5x10^6$	rhinorrhea	hRhV
16	14 mos	S	$9.7x10^6$		rhinorrhea	none
17	7 mos	A	$4.5x10^9$	hPIV: pos ($<1.0x10^3$)	pneumonia	[hPIV]
median			$3.3x10^6$			
range			$2.2x10^5$-$4.5x10^9$			

[a]C, NPA sample taken at a control visit following discharge from hospital. A, NPA taken at admission to hospital. S, NPA taken during stay in hospital. D, NPA taken at discharge from hospital. [b]hBoV, human bocavirus; hRSV, human respiratory syncytial virus; hRhV, human rhinovirus; hCoV-NL63, human coronavirus NL63. [c]*X-ray confirmed. [d]Viruses in brackets indicate a possible bystander role; other viruses indicate potential etiologic agents in association with hBoV.

TABLE 4 - *Patients with low (<1.0x10^5 DNA copies/ml) hBoV load at time of NPA collection.*

Patient #	*Age*	*Time of hBoV detection*[a]	*Viral load quantification (copies/ml NPA)*		*Clinical symptoms/syndromes*[c]	*Potential additional etiologic agent*[d]
			hBoV	*Other viruses*[b]		
18-1	3 yrs	S	2.9x10^4		pneumonia*	?
19-2	20 mos	A	3.0x10^2	hRhV: 2.5x10^6	pneumonia*	hRhV
20-3	16 mos	A	4.7x10^3	hRSV: 4.1x10^5	fever, seizures, rhinitis	hRSV-A
21-4	23 mos	A	7.2x10^3	hRSV: 1.2x10^6	pneumonia*	hRSV-B
22-5	3 yrs	A	pos (<1.0x10^3)	hRhV: 2.5x10^5 hAdV: 2.5x10^7	bronchitis*	hRhV+hAdV
23-6	2 mos	A	pos (<1.0x10^3)	hRSV: 3.7x10^5	pneumonia*	hRSV-B
24-7	2 mos	A	pos (<1.0x10^3)	hRSV: 1.4x10^6	pneumonia*	hRSV-A
25-8	8 mos	?	6.3x10^3		none	NA
26-9	2 mos	A	pos (<1.0x10^3)	hRSV: 1.0x10^5	pneumonia*	hRSV-A
27-10	7 mos	A	pos (<1.0x10^3)	hRSV: 1.8x10^5	bronchiolitis	hRSV-A
28-11	8 mos	A	pos (<1.0x10^3)	hRSV: 1.4x10^6	pneumonia*	hRSV-A
29-12	9 mos	?	3.7x10^3		none	NA
30-13	5 yrs	A	4.2x10^4	hPIV: 2.5x10^6	pneumonia*	hPIV
31-14	2 yrs	C	1.4x10^3		none	NA
32-15	3 yrs	C	2.9x10^4	hRhV: 2.5x10^4	fever	?
33-16	12 mos	C	1.4x10^4		none	NA
34-17	13 yrs	A	2.5x10^3		pneumonia*	?
35-18	14 mos	A	1.6x10^4	hRhV: 2.5x10^6	fever, cough, otalgia	hRhV
36-19	5 yrs	A	4.4x10^4		fever, cough, rhinorrhea	?
37-20	21 mos	A	3.4x10^3	hRSV: 2.5x10^3 hAdV: 2.5x10^4	rash, otitis	?
38-21	18 days	?	1.4x10^3		none	NA
39-22	22 days	S	pos (<1.0x10^3)		none	NA
40-23	1 mo	A	pos (<1.0x10^3)	hRSV: 7.9x10^5	rhinitis, cough	hRSV
41-24	27 yrs	S	pos (<1.0x10^3)		rhinorrhea, sore throat	?
42-25	42 yrs	S	4.7x10^3		none in CMLe	NA
median			1.4x10^3			
range			<1.0x10^3-4.4x10^4			

[a,b,c]See footnotes a,b and c of Table 3. hAdV, human adenovirus. [d]?, no virus was identified as a potential agent responsible for the respiratory syndrome. Control NPA, nasopharyngeal aspirate taken during a control visit a number of days after discharge from hospital. NA, not applicable (control NPA). [e]CML, chronic myelocytic leukemia.

for clinical symptoms, either alone or in association with hBoV. However, in two of these seven patients (Pt#11, and #15) high viral loads of both hBoV and hRhV were not reported to be associated to severe respiratory symptoms. The second group included ten patients. Of these, six (pts #1, #6, #8, #9, #12, and #16) had high hBoV load alone, and four (pts #4, #5, #14, and #17) showed high hBoV load in association with low viral load of other respiratory viruses. Thus, in these ten patients hBoV was the only detectable virus and, thus, could be considered the potential responsible for clinical symptoms. However, in three of these ten patients (pts #1, #9, and #16), no severe respiratory symptoms were reported.

Bystander role of hBoV infection in patients with low hBoV load

As shown in Table 4, hBoV infection was associated with low (<$1.0x10^5$ DNA copies/ml) viral load in 25/42 hBoV-positive patients (median $1.4x10^3$; range <$1.0x10^3$-$4.4x10^4$ copies/ml). Of the 25 patients, 18 displayed overt respiratory symptoms, whereas 7 were symptom-free concomitantly with negative results obtained on NPA samples taken for control. In 12 symptomatic patients, a high viral load (>$1.0x10^5$ copies/ml NPA) of one or more respiratory viruses was associated to a low hBoV load, and, thus, the hBoV-associated virus was considered potentially responsible for the episode of ARTI.

In detail, hBoV coinfecting viruses were hRSV in eight patients, hRhV alone in two patients, hRhV in association with hAdV in one patient, and hPIV in one patient. As for the other 6 symptomatic patients (pts #18-1, 32-15, 34-17, 36-19, 37-20, and 41-24), no respiratory virus or other infectious agent responsible for the ARTI episode was identified. In the remaining seven asymptomatic patients (pts #25-8, 29-12, 31-14, 33-16, 38-21, 39-22, and 42-25) a low hBoV load was associated with absence of other respiratory viruses or other infectious agents.

DISCUSSION

Following its discovery, hBoV has been associated with upper and/or lower respiratory tract infections (Allander *et al*., 2005; Manning *et al*., 2006; Bastien *et al*., 2006a; Foulongne *et al*., 2006; Ma *et al*., 2006; Sloots *et al*., 2006; Bastien *et al*., 2006b; Kupfer *et al*., 2006). In addition, recent studies found hBoV very infrequently in respiratory secretions from individuals without respiratory symptoms (Kesebir *et al*., 2006; Manning *et al*., 2006; Fry *et al*., 2007). However, in our study, hBoV was sometimes detected in subjects in the absence of both respiratory symptoms and other respiratory viruses.

It cannot be excluded that in these patients of our series hBoV detection could represent the late phase of a recent infection. It is still crucial to define, in the absence of Koch's postulates (the virus at the moment is not cultivatable, and there is no animal model available at this time), whether the virus is pathogenic or not, and, if pathogenic, when and under which circumstances it is able to express its pathogenicity (McIntosh, 2006). We tried to address this issue by quantifying hBoV load in respiratory secretions taken from patients during and, when appropriate, after the resolution of an acute episode of respiratory infection. Based on a series of studies recently conducted by our groups on acute respiratory infections caused in infants and young children by hRSV and hMPV (papers in press), we arbitrarily selected a cut-off of $1.0x10^5$ viral DNA copies/ml NPA to separate patients with high from patients with low hBoV load.

This cut-off separated all the 42 hBoV-positive patients into two groups: those (n=17) with high, and those with low (n=25) viral load. Within the group with high hBoV load, hBoV could be considered responsible for clinical symptoms (when detected alone or in association with other respiratory viruses with viral loads less than $1.0x10^5$ DNA copies/ml) or co-responsible (when associated with other respiratory viruses with viral loads greater than $1.0x10^5$ DNA copies/ml) in seven and five patients, respectively. However, in the remaining five patients high hBoV loads were not associated with overt clinical symptoms (in two cases hBoV was detected in association with hRhV, and in three cases alone). Thus, high hBoV load was sometimes associated with absence of overt clinical symptoms.

Among the patients with low hBoV load, it is important to stress that about 60% (25/42) of hBoV-positive patients had low viral load levels in respiratory secretions. In these conditions, hBoV detection may reveal residual viral shedding from a

recent infection, or may represent the initial phase of an incipient infection. In our series, this was the case in seven asymptomatic patients tested at control medical visits. For 12 of the other 18 patients with low hBoV load, the high viral load of a respiratory virus co-detected with hBoV identified with high probability the virus responsible for the current ARTI episode, whereas in the other six patients no viral or other infectious agent could be identified as responsible for respiratory symptoms.

In this study, the incidence of hBoV infections involved almost 10% of patients, while hBoV was detected in more than 12% of respiratory episodes. This is among the highest frequencies so far reported for hBoV infection in previous studies (Allander *et al.*, 2005; Kesebir *et al.*, 2006; Manning *et al.*, 2006; Arnold *et al.*, 2006; Bastien *et al.*, 2006a; Choi *et al.*, 2006; Foulongne *et al.*, 2006; Ma *et al.*, 2006; Sloots *et al.*, 2006), and is close to the frequency of 10.3% reported in a German study (Weissbrich *et al.*, 2006). In addition, our study confirms the extraordinarily high incidence of hBoV co-infections or co-detections as compared to single infections (64.4% vs 35.6%). The high rate of hBoV coinfections with other respiratory viruses in infants has been reported in all studies looking for respiratory viruses in respiratory secretions (Allander *et al.*, 2005; Manning *et al.*, 2006; Arnold *et al.*, 2006; Choi *et al.*, 2006; Foulongne *et al.*, 2006; Sloots *et al.*, 2006; Weissbrich *et al.*, 2006).

This more frequent association of hBoV infections with other respiratory infections, and particularly with hRSV infection, may suggest an exacerbating role of hBoV with respect to hRSV, but may also indicate a less pathogenic role of hBoV compared to the other viruses infecting the respiratory tract.

All cases of hBoV infection were observed in our series between December and May with peak incidence during February and March. These findings are in keeping with most data of the literature showing that the virus is seasonal and is found in fall, winter, and spring (Allander *et al.*, 2006; Kesebir *et al.*, 2006; Arnold *et al.*, 2006; Choi *et al.*, 2006). As for the hospitalized patients' age, infants and young children were about 10 times more likely to be infected by hBoV infection than adults as confirmed by all reported studies (Allander *et al.*, 2006; Manning *et al.*, 2006; Foulongne *et al.*, 2006; Ma *et al.*, 2006; Sloots *et al.*, 2006; Weissbrich *et al.*, 2006) but one (Bastien *et al.*, 2006a).

Furthermore, our study demonstrated the nosocomial transmission of hBoV infection in patients not shedding virus upon admission, but during their stay in hospital or upon discharge, as already reported in another study (Kesebir *et al.*, 2006). Finally, we could approximately determine the duration of viral shedding in patients for whom sequential sampling of respiratory secretions was possible. The median time to hBoV clearing from respiratory secretions was about one week, thus documenting that persistent viral shedding is not frequent.

The phylogenetic analysis of a fragment of VP1/VP2 genes of 22 hBoV strains confirmed clustering of 22 hBoVstrains examined into 2 genotypes, in keeping with phylogenetic data obtained in Sweden (Allander *et al.*, 2005), USA and South Africa (Kesebir *et al.*, 2006; Smuts and Hardie, 2006).

In conclusion, our study has shown a high hBoV circulation in the Italian pediatric population admitted to hospital with a 2:1 ratio of coinfections to single hBoV infections. Quantification of hBoV DNA in nasopharyngeal secretions allowed for the identification of the high replication rate of the virus in the respiratory tract. However, the pathogenic role of the virus remains to be defined in prospective studies by possibly investigating the hBoV-specific immune response. In other words, whether hBoV is a bystander virus or a coinfecting virus increasing the severity of other virus (and, particularly, hRSV) infections remains a matter for future studies.

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