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High incidence of human bocavirus infection in children in Spain

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Abstract

Background: The newly identified human bocavirus (HBoV), a member of the *Parvoviridae* family, has been associated to low respiratory tract infections in young children.

Objectives: To present the epidemiological profile and the main clinical characteristics showed by children infected with this virus in Spain.

Study design: We have studied the incidence of HBoV and other 15 respiratory viruses in 917 nasopharyngeal aspirates taken from 730 infants and children under age of 14 with acute lower respiratory tract infection from September-04 to August-06.

Results: HBoV was detected in 123 samples (13.4%) showing a seasonal distribution with November and December as the peak months. Out of the 558 samples which rendered a positive result for at least one of the virus tested, HBoV (22%) ranked fourth behind respiratory syncytial virus (181, 32%), adenoviruses (155, 28%) and rhinoviruses (136, 24%). Co-infections with HBoV and other respiratory viruses were detected in 74 out of 123 HBoV-positive specimens (60%). In addition, HBoV was also found in stool and, for the first time, in urine samples.

Conclusions: Results obtained provide further evidence that HBoV is involved in acute lower respiratory tract infections. HBoV-associated disease should not be limited to the respiratory tract.

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Keywords: *Parvoviridae*; Bronchiolitis; Pneumonia; Wheezing; Stool samples; Respiratory infection

1. Introduction

Acute lower respiratory tract infection (LRTI) refers unspecifically to infections of lower airways, either with intrapulmonary shunt (bronchitis and pneumonia) or with dynamic hyperinflation (bronchiolitis and wheeze). Respiratory syncytial virus (RSV) is clearly the major pathogen associated with LRTI during infancy and early childhood. However, parainfluenza viruses (PIV), rhinoviruses, adenoviruses, coronaviruses and influenza viruses are also

common known causative agents of LRTI in infants and children. Even so, a specific etiologic agent is often not identified in clinical practice because of lack of sensitive tests for all known agents and the presence of yet unrecognized pathogens. The recently discovered human metapneumovirus (hMPV) and human coronavirus NL63 have contributed to resolve the etiological diagnosis in many cases (van den Hoogen et al., 2001; van der Hoek et al., 2004). However, the proportion of LRTI without an etiological agent associated remains not contemptible. In 2005, human bocavirus (HBoV) was added to the ever expanding list of viruses associated to LRTI in children (Allander et al., 2005). Shortly after, a worldwide distribution of the virus was evidenced (Arnold et al., 2006; Bastien et al., 2006; Chung et al., 2006; Foulongne et al., 2006; Kaplan et al., 2006; Kesebir et al.,

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2006; Kleines et al., 2007; Lu et al., 2006; Ma et al., 2006; Sloots et al., 2006; Smuts and Hardie, 2006; Weissbrich et al., 2006).

To estimate the incidence of HBoV in children in Spain and to further define some of the epidemiological aspects and the main clinical manifestations associated with HBoV infection, we tested a total of 917 nasopharyngeal aspirate (NPA) specimens drawn from sick infants and children diagnosed of LRTI based on clinical evidence from September 2004 to August 2006. A new sensitive nested-PCR method for detection of HBoV is also described.

2. Material and methods

2.1. Clinical specimens

A total of 917 NPA specimens from 730 children under age of 14 diagnosed of acute LRTI based on clinical evidence were collected through two consecutive respiratory seasons (September-04 to August-06), irrespective of the requirement of hospitalisation and the presence of fever, at the pediatric unit of the Severo Ochoa hospital in Leganés (Madrid). Urine and stool samples were also taken from six patients during their stay in hospital in order to investigate additional routes of HBoV excretion. Specimens were aliquoted in duplicates at 200 μ l, one of them immediately processed and the remaining stored at -80°C . Specimens were screened for the presence of HBoV and other 15 respiratory viruses.

2.2. Clinical assessment

During the hospital stay pediatricians filled out a questionnaire covering sex, age (months) at admission, need for oxygen therapy measured as transcutaneous oxygen saturation, axillary temperature $\geq 38^{\circ}\text{C}$, length of hospital stay and clinical diagnosis.

2.3. Nucleic acids extraction

Nucleic acids were automatically extracted from 200 μ l aliquot of NPA specimens using the BioRobot M48 workstation and the MagAttract Virus Mini M48 Kit (Qiagen, Hilden, Germany). To check for the efficiency of acid nucleic extraction and for the presence of amplification inhibitors, an internal control was cloned and added to the lysis buffer.

2.4. Fifteen different respiratory virus detection by PCR methods

NPA specimens were tested for influenza virus A, B and C, RSV A and B and adenoviruses by using a multiplex RT-nested PCR (Coiras et al., 2003), and also for parainfluenza viruses 1–4, human coronaviruses 229E and OC43, enteroviruses and rhinoviruses by using a second multiplex RT-nested PCR (Coiras et al., 2004). hMPV was investigated

in all samples using a RT-nested PCR designed in the matrix gene (Lopez-Huertas et al., 2005).

2.5. HBoV detection by nested-PCR method

For sensitive and specific HBoV detection a nested PCR assay was developed. Primers were designed in highly conserved regions of the (NP-1) and VP1/VP2 genes based on a multiple alignment constructed with all genome sequences of bocaviruses available at the GenBank database. A whole of 5 μ l of total nucleic acid extracted were added to 45 μ l of reaction mixture containing 60 mM Tris-HCl (pH 8.5), 15 mM $(\text{NH}_4)_2\text{SO}_4$, 2 mM MgCl_2 , 200 μ M of each dNTP, 2.5 units of Taq polymerase (Amplitaq, Perkin-Elmer Cetus) and 10 pmol of primers HBOV1f (5'-CACAGGAGCMGGAGYCGCAG) and HBOV1r (5'-CCAAGATATYTRTATCCAGG) for HBoV amplification and 10 pmol of primers RTS (5'-GCTTGGGCGTGTCTCAAATCT) and RTA (5'-GTCGCCACGGTTGATGAGAGCT) for the internal control amplification. Amplifications were carried out in a Peltier Thermal Cycler PTC-200 (MJ Research). Temperature and time profiles were 94°C for 2 min, 40 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s, and a final extension at 72°C for 5 min. After the first amplification round, 1 μ l of the reaction product was added to 49 μ l of the nested reaction mixture containing the same reagents used for the first reaction mixture with exception of primers, HBOV2f (5'-GTGGTGTGGGTTCTACTGGC) and HBOV2r (5'-CTACGGTACACATCATCCCAG) for specific HBoV amplification, and NS3 (5'-CGTAATGGCTGGCCTGT) and NA2 (5'-GTAATGCTCTGCCAGTGT) for the internal control amplification. Cycling conditions were the same that those used for the first round, but only 30 cycles were necessary. A total of 10 μ l of each second round amplification product was subjected to electrophoresis on a 2% agarose in $0.5\times$ TBE gel stained with ethidium bromide (0.5 μ g/ml), and visualized under UV light. The specific band for HBoV corresponded to 243 bp and the internal control to 350 bp (Fig. 1). Usual precautions were implemented to avoid false positive results by carryover contamination. Positive results were confirmed by testing a new aliquot of sample.

Sensitivity of the HBoV nested-PCR method was estimated by testing serial dilutions of a plasmid DNA containing the first round amplified product (609 bp). This product was cloned into the pCR4-TOPO plasmid using the TOPO-TA Cloning Kit with One Shot TOP10 Chemically Competent *E. coli* (Invitrogen). Plasmids were extracted and purified using the QIAprep Spin Miniprep Kit (Qiagen). The copy number of the HBoV genome equivalents was estimated spectrophotometrically at OD_{260} .

To verify specificity, the amplified products with the expected size (243 bp) obtained after the second round of PCR were directly sequenced in both directions. PCR products were purified by using the QIAquick PCR Purification

Kit (Qiagen) and sequenced according to the manufacturer's protocol with the primers HBOV2f and HBOV2r by using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

3. Results

3.1. Sensitivity and specificity of the HBOV detection nested-PCR method

The sensitivity of the nested-PCR method for HBOV DNA detection was estimated in 10 genomic equivalents by testing serial 10-fold dilutions of the specific plasmid (Fig. 1). Specific HBOV DNA was demonstrated by sequencing all the amplification products with the expected size. Specificity of the nested-PCR assay was also assessed by testing 10 human parvovirus B19 positive serum samples.

3.2. Detection of HBOV and other 15 viruses in respiratory specimens

A positive result for HBOV was rendered in 123 out of 917 NPA specimens (13.4%) taken from 115 out of 730 patients studied (15.7%). Of these children, 49 were found to be infected only with HBOV. As shown in Table 1, recurrent wheezing or bronchiolitis were the clinical diagnosis more frequently observed. Remarkably, three distinct patients presented two independent HBOV-positive NPA samples submitted apart 28, 35, and 150 days, respectively.

The monthly distribution of HBOV infections in both seasons studied is shown in Fig. 2. HBOV was detected through the entire season with November and December as the peak months. No HBOV-positive specimens were obtained during July and August.

In addition to HBOV, all samples were examined for the presence of other 15 different respiratory viruses. Out of the 917 NPA specimens studied, at least one respiratory virus was detected in 558 samples (61%): RSV-A and RSV-B 181

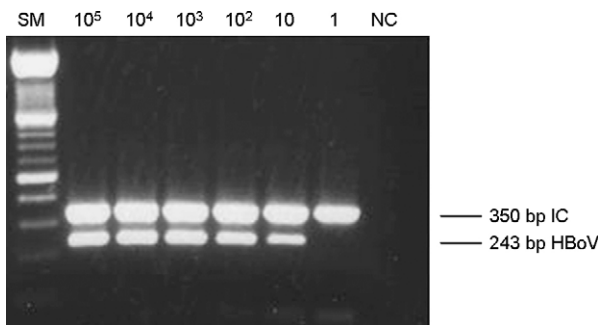


Fig. 1. Ethidium bromide stained agarosa gel showing the specific 243 bp HBOV amplification product and the 350 bp band size corresponding to the internal control (IC). Sensitivity limit was estimated in 10 genomic equivalents by testing serial 10-fold dilutions of the specific plasmid. SM: size markers (100 bp). NC: negative control (H₂O).

Table 1

Characteristics of 49 children infected with HBOV as a sole respiratory virus

Characteristic	Value
Demographic data	
Age (mo), median (range)	22 (4–52)
Sex, male:female	29:20
Clinical characteristic	
Hospitalization	43 (88%)
Duration, median days \pm S.D.	4.3 \pm 2.8
Hypoxia (Sat. O ₂ \leq 93%), n = 43 ^a	25 (58%)
Clinical diagnosis	
Recurrent wheezing	22 (45%)
Bronchiolitis	12 (24%)
Pneumonia	7 (14%)
Fever syndrome	4 (8%)
Upper respiratory infection	4 (8%)

^a Hypoxia was only determined in hospitalized children.

(32%), adenoviruses 155 (28%), rhinoviruses 136 (24%) and HBOV that ranked fourth with 123 (22%) positive samples. Other different viruses detected were, in descending order of frequency, influenza A virus (33, 6%), hMPV (35, 6%), PIV-3 (28, 5%), enteroviruses (16, 3%), PIV-1 (14, 3%), PIV-4 (13, 2%), PIV-2 (9, 2%), influenza B virus (6, 1%) and coronavirus 229E (5, 1%).

Co-infections between HBOV and other respiratory viruses were detected in 74 out of 123 HBOV-positive specimens (60%). Dual detection was mainly observed with adenoviruses in 20 samples (27%), with RSV in 17 (23%) and rhinoviruses in 12 (16%). Three or more viruses were detected in 18 cases (24%).

3.3. Detection of HBOV in urine and stool specimens

HBOV DNA was additionally detected in urine and stool samples taken from two long-hospitalized (>5 days) children which had previously presented HBOV positive result in respiratory samples. No digestive problems, diarrhea or abdominal pain were observed in these patients.

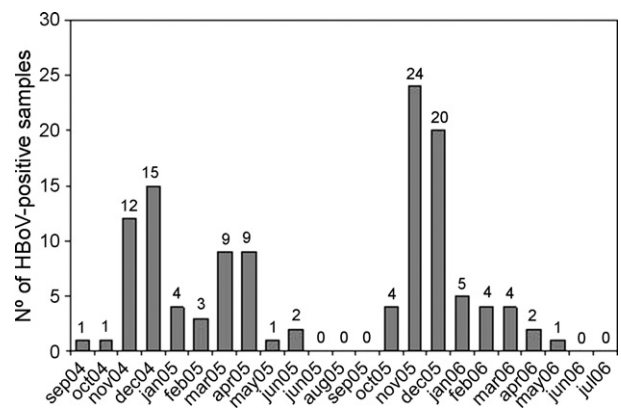


Fig. 2. Monthly distribution of HBOV-positive specimens from September 2004 to July 2006.

3.4. Single-nucleotide polymorphisms in HBoV amplification products

A total of 52 selected sequences of HBoV amplified products were further analysed. Compared with the reference isolate ST1 (GenBank DQ000495), an identical sequence was obtained in 38 isolates. A second group comprised seven sequences contained a G → A change at nucleotide 2744, identical to that observed in ST2 isolate (DQ000496). A third group of six sequences were identical to the Chinese isolate CZ745 (DQ494204) showed an A → G change at nucleotide 2786. Finally, a single-nucleotide polymorphism was observed in a single Spanish isolate, showing a G → A change at nucleotide 2804. None of these three nucleotide changes resulted in changes in the predicted amino acid sequence. Four of the sequences obtained were submitted to the GenBank database under the accession numbers EF117275–EF117278.

4. Discussion

In addition to well-established pathogens, novel viruses associated to respiratory infections have been identified in recent years, including hMPV (van den Hoogen et al., 2001) and three different coronaviruses (Peiris et al., 2003; van der Hoek et al., 2004; Woo et al., 2005). All of them are members of well recognized families of viruses involved in respiratory infections, such as *Paramyxoviridae* and *Coronaviridae*. For the first time, a member of the family *Parvoviridae*, HBoV, included in the genus *Bocavirus*, has also been detected in patients suffering respiratory illness (Allander et al., 2005).

In this study, the overall incidence of HBoV and other 15 different respiratory viruses among previously healthy children with acute LRTI was evaluated by using different nucleic acid amplification assays. Out of the 917 NPA specimens studied, a total of 123 tested positive for HBoV, accounting for 13.4%. This high incidence of HBoV infection observed in our study population, which consisted mainly in hospitalized patients, are in accordance with rates previously published ranging from 10.3% to 19% (Allander et al., 2007; Choi et al., 2006; Kaplan et al., 2006; Kleines et al., 2007; Smuts and Hardie, 2006; Weissbrich et al., 2006). Nevertheless, rates appreciably lower ranging between 3.1% and 8% have also been published by other authors (Allander et al., 2005; Arnold et al., 2006; Chung et al., 2006; Foulongne et al., 2006; Kesebir et al., 2006; Lu et al., 2006; Ma et al., 2006; Sloots et al., 2006). Rather than geographic differences in HBoV circulation, this discrepancy may be explained by differences observed in the patients studied. Several studies included exclusively hospitalized children while others enrolled either hospitalized and outpatient children. In the same way, several studies are focused on LRTI patients and others are based on patients with less severe lung disease, or infants and young children versus adults patients. In addition, differences observed in the incidence of HBoV infection

could also be due to the variety of respiratory samples taken from patients, or alternatively to differences inherent in sensitivity of PCR assays employed.

The sensitivity of the nested-PCR assay we have developed is high enough to detect at least 10 copies of HBoV genome equivalents. Comparable sensitivity has been reported using real-time PCR protocols (Allander et al., 2007; Kleines et al., 2007; Lu et al., 2006). Allander et al. detected HBoV in 49 NPA specimens out of 259 (19%) children hospitalized for acute expiratory wheezing. This percentage represents the highest incidence of HBoV infection reported to date. Interestingly, Kleines et al. quantified HBoV in samples and revealed that low viral loads dominated, irrespective of severity of clinical symptoms or patient's age. This finding highlights the importance of using very sensitive PCR assays to warrant the detection of HBoV. The good sensitivity of the nested-PCR assay used in this study permitted, as well as in real-time PCR protocols, high rates of HBoV detection.

Our data reveals the presence of HBoV in stool and, for the first time, in urine specimens of two patients with respiratory symptoms. No digestive problems, diarrhea nor abdominal pain were observed in these patients. Nevertheless, diarrhea is a relatively common symptom presented in patients with HBoV infection (Arnold et al., 2006; Kesebir et al., 2006). The presence of HBoV in stool and urine samples may merely reflect the excretion of the virus by the digestive and urinary tracts, but also could be considered suggestive of other manifestations of HBoV-associated disease, not limited to the respiratory tract. As a support of this hypothesis, detection of HBoV in serum specimens taken from children with an episode of acute wheezing has recently been published (Allander et al., 2007). Systemic infections are a well-known fact for most parvovirus infections, including both bovine parvovirus (Storz et al., 1978) and minute virus of canines (Carmichael et al., 1994), the two other members of the genus *Bocavirus*.

The monthly distribution of HBoV-positive specimens suggests an epidemiological profile similar to adenoviruses and rhinoviruses, with cases reported throughout the entire epidemic season, in contrast to RSV, hMPV and influenza A and B viruses, with most of the cases reported in the context of outbreaks during the winter cold months. In our Mediterranean country, HBoV infections were identified in September to June over a continuous 23-month period studied (Fig. 2). November and December were by far the peak months, in contrast to those reported in Korean children occurring from May to July (Choi et al., 2006).

It is pointed up the common occurrence of multiple detection of respiratory viral agents, which suggest the high incidence of co-infections in respiratory infection. In this study, HBoV was co-detected with one or more respiratory viruses in 74 out of 123 HBoV-positive specimens (60%). This high co-infection rate found in our patients is agreed with other series published (Allander et al., 2005; Choi et al., 2006; Sloots et al., 2006). High co-infections rates is not in opposition to a pathogenic role of HBoV in respiratory dis-

eases. In fact, the only study to date including asymptomatic control infants reported a zero incidence of HBoV infection in this group, then providing a statistical association of the virus with disease (Kesebir et al., 2006).

Remarkably, three distinct patients presented two different HBoV-positive NPA specimens submitted apart 28, 35, and 150 days, respectively. This could suggest a long-time persistence of HBoV after initial acute infection, a possibility strongly supported by the evidence of HBoV DNA presence in convalescent-phase serum specimens collected from children with acute wheezing 2–3 weeks after discharge from the hospital (Allander et al., 2007). Nevertheless, it might be a reflect that infants and children, as well as occurs with other respiratory viruses, could be re-infected with HBoV, twice at least in the same season. Unfortunately, urine and stool specimens could not be collected from these three patients.

Taken together, these results provide further and convincing evidence that HBoV is involved in LRTI. Additional studies will clarify the clinical and epidemiological significance of the presence of HBoV in clinical samples other than respiratory origin, such as urine and stool specimens.

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