Human Bocavirus Isolated From Children With Acute Respiratory Tract Infections in Korea, 2010–2011

Jong Gyun Ahn,1,2 Seong Yeol Choi,3 Dong Soo Kim,2,3 and Ki Hwan Kim2,3*

1Department of Pediatrics, School of Medicine, Ewha Womans University, Seoul, Korea
2Department of Medicine, The Graduate School of Yonsei University, Seoul, Korea
3Department of Pediatrics, Severance Children’s Hospital, Yonsei University College of Medicine, Seoul, Korea

INTRODUCTION

Over the past decade advancement of new molecular techniques has led to the detection of various potential respiratory viruses such as human metapneumovirus (HMPV) [van den Hoogen et al., 2001], human coronavirus (HCoV) NL63 [van der Hoek et al., 2004] and HKU1 [Woo et al., 2005], severe acute respiratory syndrome-coronavirus (SARS-CoV), [Drosten et al., 2003], and human bocavirus (HBoV) [Allander et al., 2005]. HBoV was first isolated in 2005 from nasopharyngeal specimens from children with respiratory tract infections [Allander et al., 2005]. HBoV, a small non-enveloped virus with an approximately 5 kb single-stranded DNA genome is classified in the family Parvoviridae, subfamily Parvovirinae, and genus Bocavirus. Since it was first identified, HBoV has been detected worldwide in respiratory samples. Several studies have shown that HBoV is associated with acute respiratory tract infections in young children, with a reported worldwide prevalence ranging from...
1.5% to 19% [Bastien et al., 2006; Allander et al., 2007; Fry et al., 2007].

In recent years, three closely related variants of HBoV have been reported, designated as HBoV2, HBoV3, and HBoV4 [Arthur et al., 2009; Kapoor et al., 2009, 2010]. HBoV2 was discovered in the stool of children with non-polio acute flaccid paralysis in Pakistan [Kapoor et al., 2009]. Soon thereafter, HBoV3 and HBoV4 were identified in the stools of children with diarrhoea in Australia and the USA [Arthur et al., 2009; Kapoor et al., 2010]. To date, HBoV1 has been linked most frequently with respiratory tract illness, particularly in children [Bastien et al., 2006; Kesebir et al., 2006; Allander et al., 2007; Fry et al., 2007; Maggi et al., 2007], although it has also been detected in stool samples to a lesser extent [Albuquerque et al., 2007; Lau et al., 2007; Lee et al., 2007]. In contrast, HBoV2 occurs rarely in respiratory samples, and instead is found primarily in the stools of children, indicating an association between HBoV2 and gastrointestinal disease [Arthur et al., 2009; Chieochansin et al., 2009; Han et al., 2009; Kapoor et al., 2009; Chow et al., 2010; Santos et al., 2010]. Interestingly, the identification of HBoV3 and HBoV4 is so recent that the epidemiology and clinical characteristics associated with these viruses are only now being realized [Arthur et al., 2009; Chieochansin et al., 2009; Kapoor et al., 2010; Santos et al., 2010].

The clinical significance and molecular characteristics of these newly identified HBoV species have not been completely assessed. The aim of this study was to investigate the presence of HBoV infection and to investigate the presence of HBoV in the nasopharyngeal aspirates of children with acute respiratory tract infections between 2010 and 2011.

**Materials and Methods**

**Study Patients and Specimen Collection**

Between January 2010 and December 2011, 1,528 nasopharyngeal specimens were collected from children hospitalized with acute respiratory tract infections at Severance Children’s Hospital in Seoul, Korea. As part of the diagnosis, pneumonia, croup, bronchiolitis, asthma, acute pharyngotonsillitis, acute nasopharyngitis, and acute respiratory distress syndrome (ARDS) were included, which were diagnosed by the physician through physical examination and chest X-rays according to the World Health Organization (WHO) recommended guidelines [World Health Organization, 2005]. Patients who had immunocompromised conditions (e.g., current treatment of chemotherapy or immunosuppressive therapy, bone marrow transplantation or known primary immune deficiency) were all excluded from the study. Nasopharyngeal aspirates were obtained by suction using a fine, flexible plastic catheter and syringe and then immediately transported at 4°C to the laboratory, where they were stored at −70°C until use. Review of medical records, interviews at admission, and patient demographic and clinical data including medical history, detailed signs and symptoms, physical examination, and laboratory and radiology results were all collected. Disease severity was assessed according to a respiratory symptom scoring system based on a previously published metric [Gern et al., 2002; Lemanske et al., 2005].

**Multiplex PCR/RT-PCR for HBoV and Other Respiratory Viruses**

Viral DNA or RNA from each nasopharyngeal sample was extracted with a QIAamp Viral Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer’s protocol. A one-step Multiplex PCR/RT-PCR kit (SolGent, Daejeon, Korea) was used to detect 12 respiratory viruses [Chun et al., 2009], including HBoV, respiratory syncytial virus (RSV), human rhinovirus (HRV), influenza A virus (IAV), influenza B virus (IBV), HMPV, adenovirus (ADV), HCoV 229E, OC43, and parainfluenza viruses (PIV)1–3. Amplification was performed according to the manufacturer’s instructions, and products were run on a 2% agarose gel, stained with ethidium bromide, and visualized by UV light.

**Sequencing of VP1/2 and Phylogenetic Analysis**

HBoV-positive specimens were amplified with nested PCR using pan-bocavirus PCR primers targeting the VP1/VP2 gene as described previously [Kapoor et al., 2010]. Positive PCR samples were sequenced by SolGent Co. (Daejeon, Korea). VP1/VP2 sequences were aligned by CLUSTAL W and phylogenetic analysis was conducted with MEGA 5.0 software. A topology tree was drawn by the neighbour-joining method and Kimura 2-parameter model bootstrapped with 1,000 replications.

**Statistical Analysis**

Statistical analysis was performed with SPSS (version 18.0, Chicago, IL). Values are expressed as percentages for discrete variables, or as the mean (m) ± standard deviation (SD) for continuous variables. Clinical features and laboratory variables were analyzed using the Pearson χ² test, Fisher’s exact test, or Student’s t-test. P-values less than 0.05 were considered statistically significant.

**Results**

**Epidemiologic Characteristics of HBoV**

HBoV was detected in 187 (12.2%) of 1,528 nasopharyngeal aspirates. Overall, HBoV was the fourth most frequent pathogen identified in the study patients, following HRV (22.9%), ADV (20.9%), and RSV (17.4%) (Fig. 1). A total of 107 (57.2%) HBoV-positive
samples were co-detected with other respiratory viruses. One additional virus was found in 73.8%, two in 22.4%, and three in 3.7%. HRV, ADV, and HMPV were the most frequently co-detected (Table I).

Seasonal distribution of HBoV was noted between March and December, with a peak occurring in the month of June in both 2010 and 2011 (Fig. 2). Of the 187 HBoV-positive patients, 112 (59.9%) were male and 75 (40.1%) were female, with no significant difference between HBoV positive and negative patients (Table II). The mean ± SD age of HBoV infected children (24.3 ± 15.9 months) was significantly less than that of the HBoV-negative group (31.5 ± 30.5 months) (Table II). Further, the group aged 12 to <24 months had the highest rate of HBoV infection (43.9%), while the group >60 months of age had the lowest rate (3.2%) (Fig. 3).

Clinical Features of HBoV-Positive Patients

Of the 187 cases of HBoV infection, the main diagnosis was pneumonia (65.8%), followed by bronchiolitis (20.3%), croup (5.9%), asthma (3.7%), acute pharyngotonsillitis (2.7%), acute nasopharyngitis (1.1%), and ARDS (0.5%).

Previous wheezing history ($P = 0.016$), frequency of chest retraction and wheezing ($P < 0.001$ and $P = 0.022$, respectively), respiratory symptom score

| Type of infection | Virus        | No. of patients (%) | Subtotal (%) |
|------------------|--------------|---------------------|--------------|
| Single infection | HBoV (only)  | 80 (42.8)           | 80 (42.8)    |
| Co-infection     | HBoV ADV     | 39 (20.9)           | 107 (57.2)   |
|                  | HBoV HRV     | 16 (8.6)            |              |
|                  | HBoV ADV     | 10 (5.3)            |              |
|                  | HBoV ADV RSV | 7 (3.7)             |              |
|                  | HBoV PIV3    | 4 (2.1)             |              |
|                  | HBoV ADV RSV | 3 (1.6)             |              |
|                  | HBoV HRV HMPV| 3 (1.6)             |              |
|                  | HBoV PIV1    | 2 (1.1)             |              |
|                  | HBoV ADV PIV3| 2 (1.1)             |              |
|                  | HBoV ADV RSV HMPV | 2 (1.4) |              |
|                  | HBoV ADV HMPV| 1 (0.5)             |              |
|                  | HBoV ADV HCoV (OC43)| 1 (0.5) |              |
|                  | HBoV HMPV HCoV (229E)| 1 (0.5) |              |
|                  | HBoV HCoV (OC43)| 1 (0.5)             |              |
|                  | HBoV HRV IBV | 1 (0.5)             |              |
|                  | HBoV PIV3 HRV| 1 (0.5)             |              |
|                  | HBoV RSV HCoV (OC43)| 1 (0.5) |              |
|                  | HBoV ADV HRV HMPV | 1 (0.5) |              |
|                  | HBoV ADV HRV PIV1| 1 (0.5) |              |
| Total            |              | 187 (100)           |              |

HBoV, human bocavirus; HRV, human rhinovirus; HMPV, human metapneumovirus; ADV, adenovirus; RSV, respiratory syncytial virus; PIV, parainfluenza virus; HCoV, human coronavirus; IBV, influenza virus type B.
**TABLE II. Clinical and Laboratory Characteristics of HBoV Positive Patients**

| Clinical data                          | HBoV (+), n = 187 | HBoV (−), n = 1,341 | P-value |
|----------------------------------------|-------------------|---------------------|---------|
| Age (months)                           | 24.3 ± 15.9       | 31.5 ± 30.5         | <0.001* |
| Gender (male%)                         | 59.9              | 60.3                | 0.925   |
| Prematurity Hx. (%)                    | 10.7              | 8.4                 | 0.303   |
| Congenital heart disease Hx. (%)       | 4.8               | 5.7                 | 0.606   |
| Neurologic disorder Hx. (%)            | 10.7              | 7.6                 | 0.144   |
| Previously wheezing Hx. (%)            | 19.8              | 13.3                | 0.016*  |
| Chest retraction (%)                   | 24.1              | 11.9                | <0.001* |
| Wheezing (%)                           | 40.1              | 31.7                | 0.022*  |
| Tachypnea (%)                          | 2.7               | 1.2                 | 0.103   |
| Respiratory symptom score              | 8.5 ± 4.1         | 7.5 ± 3.4           | 0.002*  |
| WBC (×10^3 cells/mm)                   | 11.4 ± 5.0        | 11.1 ± 5.3          | 0.501   |
| Haemoglobin (g/dl)                     | 12.0 ± 1.1        | 12.0 ± 1.1          | 0.986   |
| Platelet count (×10³ cells/mm)         | 380.4 ± 131.2     | 373.4 ± 139.4       | 0.544   |
| Neutrophil (%)                         | 53.2 ± 16.6       | 51.7 ± 19.4         | 0.289   |
| Lymphocyte (%)                         | 34.7 ± 15.0       | 36.0 ± 17.3         | 0.342   |
| CRP (mg/L)                             | 21.3 ± 33.7       | 26.9 ± 40.1         | 0.088   |
| ESR (mm/hr)                            | 32.8 ± 26.4       | 36.0 ± 25.7         | 0.137   |
| Total protein (g/dl)                   | 6.5 ± 0.5         | 6.5 ± 0.5           | 0.103   |
| Albumin (g/dl)                         | 4.2 ± 0.4         | 4.2 ± 0.5           | 0.149   |
| ALT (IU/L)                             | 17.8 ± 16.6       | 21.6 ± 23.9         | 0.011*  |
| AST (IU/L)                             | 32.9 ± 11.5       | 36.4 ± 20.6         | 0.001*  |
| Length of hospital stay (days)         | 4.7 ± 5.2         | 3.8 ± 4.6           | 0.021*  |

*P < 0.05.

(P = 0.002), and length of hospital stay (P = 0.021) were significantly higher in the HBoV-positive group than in HBoV-negative children (Table II). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in both groups were within normal ranges, although there was a significant difference between them (P = 0.011 and P = 0.001, respectively) (Table II). Aside from ALT and AST, no significant differences were observed with respect to underlying history such as prematurity, congenital heart disease and neurologic disorder, frequency of tachypnea, and laboratory findings between HBoV positive and negative patients (Table II).

Between children in which HBoV was the only specimen detected and those co-infected with both HBoV and other viruses, there were no significant differences in clinical and laboratory data other than gender ratio (P = 0.017), the percentage of neutrophils in white blood cells (WBCs) (P = 0.037), and total protein (P = 0.036) (Table III). However, the neutrophil ratio in WBCs and total protein were within the normal range in both groups.

**Phylogenetic Analysis of HBoV**

To investigate the divergence of the HBoV genome, a 576-bp fragment of the VP1/VP2 gene (nucleotides 3328–3903) was sequenced for all HBoV-positive samples. Phylogenetic analysis demonstrated that all of 187 HBoV-positive strains were HBoV 1, and HBoV 2, HBoV 3, and HBoV 4 were not detected (Fig. 4). There was a high nucleotide identity (98.78–100%) between all HBoV sequences. As most of the nucleotide variations were conservative at the amino acid level, there was a 98.96–100% sequence identity among all strains. Overall, when compared to the Swedish reference strain (accession number NC_007455), 17 of the 576 nucleotide positions were found to be variable, with 15 (88.2%) transitions and 2 (11.8%) transversions. No significant differences were observed in clinical and laboratory data between the group with nucleotide variations and those with wildtype HBoV1.

**DISCUSSION**

Since the first report of HBoV in 2005, it has been detected frequently worldwide in respiratory samples, with a reported prevalence ranging from 1.5% to 19% [Schildgen et al., 2008; Tran et al., 2013]. In Korea, several reports on the presence of HBoV have been published, with a frequency range of 4.7–19.1% [Choi et al., 2006, 2007, 2011; Chung et al., 2006; Kim et al., 2011]. In the present study, HBoV was detected in 187 (12.2%) of 1,528 respiratory samples. The difference in frequency in the present study and...
those previously reported may be related to the different study populations and periods. Nevertheless, this study confirmed that HBoV is circulated frequently in Korean children with acute respiratory tract infections, and was the fourth most prevalent virus among the 12 respiratory viruses which were tested.

Previous studies have reported that the seasonal preference of HBoV infections varies among different counties [Schildgen et al., 2008]. These differences are thought to be due to differences in climates and various factors affecting the prevalence of respiratory infections in each country. In this study, the peak of HBoV infections occurred during the month of June in both 2010 and 2011. These results were similar to those of two earlier studies [Choi et al., 2006; Kim et al., 2011] in Korea that reported HBoV infections peaked in May and June between 2000 and 2005 and between April and June in 2006. In close accordance with previous studies, this study suggested that HBoV was especially prevalent in the late spring and early summer seasons in Korea.

The age distribution data in this study showed that the mean ± SD age was significantly younger in HBoV-positive patients compared with HBoV-negative patients, with a peak incidence of HBoV observed in children aged 12 to <24 months and lowest incidence in children older than 60 months. These results are in close agreement with numerous previous studies [Kesebir et al., 2006; Fry et al., 2007; Maggi et al., 2007], which showed that most patients with HBoV infections are infants or children below 5 years of age. A previous seroepidemiologic study of HBoV showed that the seropositive rate in children aged under 3 months is 90.5%, but drops to 5.6% in children 6–8 months old, likely due to waning levels of maternally-acquired antibodies [Endo et al., 2007]. The percentage of HBoV-seropositive patients rebounds and increases with age, such that all children are exposed to HBoV by the age of six. One study [Kahn et al., 2008] showed that although 91.8% of infants ≤2 months old are HBoV-seropositive, the seropositive rate is lowest (25.0%) in children aged 4 months, but is increased to >85% in children ≥48 months old. Together, these data suggest that infants younger than 4–6 months of age may be protected from HBoV infection by maternal antibodies, and that the first HBoV infection begins after the loss of maternal antibodies, with the majority of HBoV infections occurring very early in childhood. In addition, the lower rate of detection of HBoV in older children and adults, as reported in several studies [Allander et al., 2005, 2007; Fry et al., 2007; Lau et al., 2007; Han et al., 2009; Noh et al., 2013], may be due to life-long immunity acquired from an infection early in childhood.

With respect to the clinical features of HBoV infection, the present study showed that HBoV-positive children had a higher prevalence of prior wheezing history, a higher frequency of chest retraction and wheezing, higher respiratory symptom scores, and a longer duration of hospital stay compared with the HBoV-negative group. These results are in agreement with previous studies reporting an association of HBoV with acute wheezing in children [Choi et al., 2006; Chung et al., 2006; Allander et al., 2007; Fry et al., 2007; Deng et al., 2012] as well as respiratory distress and hypoxia as main clinical characteristics of HBoV infection [Moriyama et al., 2010]. In addition, several studies have shown that HBoV is a cause of severe exacerbation of asthma in children [Naghipour et al., 2007; Vallet et al., 2009]. Although
the impact of co-infection with other viruses should be considered, results of this study showed that there were no significant differences in clinical features between children with HBoV infection alone and those co-infected with another virus, implying that HBoV-positive patients experience clinical severities such as respiratory distress, wheezing, and longer hospitalization regardless of co-infection status. Deng et al. [2012] suggested that a high HBoV viral load could lead to longer duration of wheezing and hospitalization in children with severe lower respiratory tract infection, while co-infection does not influence clinical severity and outcome of HBoV infection.

In the present study, HBoV2, HBoV3, and HBoV4 in respiratory samples was not detected. HBoV1 is well described in association with respiratory illness. Although recent reports have described an HBoV2-4 prevalence of 0.4–4.3% in respiratory specimens children [Han et al., 2009; Song et al., 2010; Koseki et al., 2012], the roles of HBoV2-4 in respiratory disease remain unclear. HBoV2-4 occurs rarely in respiratory samples but is fairly common in stools of children [Arthur et al., 2009; Chieochansin et al., 2009; Han et al., 2009; Kapoor et al., 2009, 2010; Chow et al., 2010; Santos et al., 2010], suggesting an association between HBoV2-4 and gastrointestinal disease. Results of the present study indicate that HBoV2-4 is not directly involved in respiratory tract infections. Further, it is presumed that HBoV2-4 is detected rarely in respiratory secretions originating from gastrointestinal mucosa due to contamination with oral secretions at the time of sampling, although

**Fig. 4.** Phylogenetic analysis of the VP1/VP2 gene junction sequence in HBoV isolates. The topology tree was constructed by the neighbour-joining method using MEGA 5.0. Black dots designate reference strains; other sequences were analyzed in the present study. GenBank accession nos. HBoV2 PK-5510 (FJ170278); HBoV3 W471 (EU918736); HBoV4-NI-385 (FJ973561); LVK (GU338055); TW2715 06 (EU984232); HK4 (EF450720); HK24 (EF450740); HK11 (EF450727); st1 (HBoV strain st1, DQ000495); CU49 (EF203921; CZ643 (DQ457413); CRD2 (DQ340570); st2 (HBoV strain st2, DQ000496).
further studies are needed to determine if HBoV2-4 are associated with clinical illness outside of the gastrointestinal tract.

Phylogenetic analysis in this study confirmed the results of previous findings [Allander et al., 2005; Chung et al., 2006; Kesebir et al., 2006; Lau et al., 2007; Neske et al., 2007] showing that HBoV1 is highly conserved with a very low degree of genetic variability. Based on these results, Lau et al. [2007] predicted that HBoV infections may occur only once because of the subsequent development of life-long immunity conferred by neutralizing antibodies. In addition, it is postulated that HBoV1-associated respiratory infections might have a homogenous pattern of clinical and epidemiologic features because of the low genetic diversity of HBoV1. In support of this possibility, the study results indicated that there were no significant differences with respect to both clinical and laboratory data between children with and without minor HBoV1 variations. Specifically, even though 23 strains with nucleotide variations out of 187 HBoV isolates was identified, only 2 of the 23 strains resulted in an amino acid substitution. Thus, the 21 strains with nucleotide variations were thought to have the same biologic characteristics as non-mutated HBoV. In addition, the two strains with an amino acid substitution did not exhibit specific clinical features, suggesting they may not influence the biological action of HBoV. In contrast, several novel types of HBoV2-4, which were not detected in this study, exhibit a high degree of genetic diversity relative to HBoV1 [Arthur et al., 2009; Kapoor et al., 2009, 2010]. These findings suggest that HBoV2-4 and HBoV1 have different clinical impacts and support the above proposal that HBoV2-4 are not directly involved in respiratory disease.

This study had several limitations. First, the study patients were limited to children hospitalized with respiratory infections at a single-centre. Second, quantification of viral loads, which would be useful to analyze co-infection cases more clearly, was not measured. Third, there is the lack of an asymptomatic control group. Fourth, the evaluation of the causative agents of acute respiratory infections covered only 12 respiratory viruses, and other bacterial or viral pathogens which can cause acute respiratory illnesses were not investigated. Despite these shortcomings, these data should aid in the understanding of the role that HBoV plays in respiratory illness.

In summary, HBoV1 with minimal sequence variations circulated in children with acute respiratory infections between 2010 and 2011, and was associated with several clinical characteristics including age, seasonality, and clinical severity with retraction, wheezing, and longer hospitalization.

It is concluded that HBoV1 is an important respiratory pathogen in young children and exhibits a homogenous pattern of clinical and epidemiologic features due to the relatively minor degree of genetic variation. Further studies are needed to clarify the clinical relevance of the minimal sequence variations within HBoV1 and to define the clinical roles of HBoV2-4.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance rendered by Ms. Kim Heui Og.

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