

# Human Bocavirus in Children With Acute Respiratory Infections in Vietnam

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Acute respiratory infections are the major cause of morbidity and mortality globally. Human bocavirus (HBoV), a novel virus, is recognized to increasingly associate with previously unknown etiology respiratory infections in young children. In this study, the epidemiological, clinical, and molecular characteristics of HBoV infections were described in hospitalized Vietnamese pediatric patients. From April 2010 to May 2011, 1,082 nasopharyngeal swab samples were obtained from patients with acute respiratory infections at the Children's Hospital 2, Ho Chi Minh City, Vietnam. Samples were screened for HBoV by PCR and further molecularly characterized by sequencing. HBoV was found in 78 (7.2%) children. Co-infection with other viruses was observed in 66.7% of patients infected with HBoV. Children 12–24 months old were the most affected age group. Infections with HBoV were found year-round, though most cases occurred in the dry season (December–April). HBoV was possible to cause severe diseases as determined by higher rates of hypoxia, pneumonia, and longer hospitalization duration in patients with HBoV infection than in those without ( $P$ -value <0.05). Co-infection with HBoV did not affect the disease severity. The phylogenetic analysis of partial VP1 gene showed minor variations and all HBoV sequences belonged to species 1 (HBoV1). In conclusion, HBoV1 was circulating in Vietnam and detected frequently in young children during dry season. Acute respiratory infections caused by HBoV1 were severe enough for hospitalization, which implied that HBoV1 may have an important role in acute respiratory infections among children. *J. Med. Virol.* 86:988–994, 2014. © 2013 Wiley Periodicals, Inc.

**KEY WORDS:** epidemiological; clinical; molecular characteristics; bocavirus

## INTRODUCTION

Acute respiratory infections are the leading cause of morbidity and mortality among children worldwide. Viruses are responsible for most of acute respiratory infections. Up to now, many viruses have been identified as the main cause of acute respiratory infections, such as respiratory syncytial virus, human metapneumovirus, influenza virus, parainfluenza virus, coronavirus, and adenovirus. However, a rather high percentage of acute respiratory infections still remains unknown the etiology. In recent years, several newly discovered viruses were reported to have association with respiratory infections, including human bocavirus (HBoV) [Mahony, 2008].

In 2005, HBoV was first identified in nasopharyngeal aspirate of children with acute respiratory infections in Sweden [Allander et al., 2005]. This novel virus belongs to the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Bocavirus* with the single-stranded linear DNA genome of about 5.3 kb. The genome contains three open reading frames encoding two non-structural proteins, NS1 and NP1, as well as two structural capsid proteins, VP1 and VP2. Up to now, four species of HBoV have been identified, namely HBoV1, HBoV2, HBoV3, and HBoV4. HBoV1 is predominantly detected in respiratory samples. On

Grant sponsor: Grants-in-Aid from the Ministry of Education and Sciences; Grant sponsor: Ministry of Health, Labor and Welfare, Japan

Conflict of interest: None declared.

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Accepted 3 September 2013

DOI 10.1002/jmv.23789

Published online 12 October 2013 in Wiley Online Library (wileyonlinelibrary.com).

the contrary, HBoV2–4 have been found mainly in stool samples [Jartti et al., 2012].

Since its first discovery, HBoV has been widely detected in many countries. HBoV was mainly found in children with acute respiratory infections at the rate ranging from 1.5% to 19% [Malecki et al., 2011; Jartti et al., 2012], indicating that HBoV is the third most common respiratory virus, after respiratory syncytial virus and human rhinovirus. However, in many epidemiological studies, HBoV was co-detected frequently with other respiratory pathogens, as well as often seen in asymptomatic children, which raising the question about its causality. Despite of that, recent identification of HBoV in blood samples together with advances in serodiagnosis have provided the increasing evidence for a causal role of HBoV in respiratory illness [Jartti et al., 2012]. Most of the published studies so far emphasized on the prevalence and epidemiology of HBoV. Only a few reports provided data related to clinical characteristics of HBoV infection. Also, the epidemiology of respiratory viruses seems to have the geographic and temporal characteristics.

This study was conducted on respiratory specimens obtained from children admitted to hospital with acute respiratory infections during a 14-month period in Vietnam to investigate the epidemiological features and specify the clinical characteristics of HBoV infections. A phylogenetic analysis of HBoV was also carried out.

## MATERIALS AND METHODS

### Patients and Samples

From April 2010 to May 2011, 1,082 children younger than 15 years old who were hospitalized with acute respiratory infections [WHO, 2005] at the Children's Hospital 2, Ho Chi Minh City, Vietnam, were enrolled in the study. The study was approved by the Scientific and Ethical Committee of the Children's Hospital 2. The written consent was obtained from the parent or legal guardian of the participants. Patients who had underlying chronic diseases (e.g., bronchopulmonary dysplasia, congenital heart disease, and immunodeficiency), or coexisting acute systemic illnesses (e.g., sepsis), or proven or suspected non-infectious respiratory symptoms (e.g., asthma), were all excluded from the study. Demographic and clinical data were recorded on a standardized questionnaire. Acute respiratory infections with the presence of an infiltrate on chest X-ray were categorized as pneumonia. Bronchiolitis was defined as under-2-year-old children presenting with wheezing and hyperaeration, atelectasis, or peribronchial thickening on chest X-ray. Croup was characterized by hoarseness, cough, and stridor. Upper respiratory tract infection (URTI) was defined as acute respiratory infections with no abnormalities on chest X-ray.

Nasopharyngeal flocked swabs (MicroRheologics, Brescia, Italy) were obtained from all enrolled children within 24 hr after admission. The specimens

were immediately placed in tubes containing 2 ml sterile physiological saline fluid and stored at  $-20^{\circ}\text{C}$  until further analysis at the laboratory.

### Virus Detection

Viral genomes were extracted directly from the specimens using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and stored at  $-80^{\circ}\text{C}$ . HBoV was detected by PCR method using pan-bocavirus primers as described previously [Kapoor et al., 2010]. Briefly, a pair of primers AK-VP-F1 (5'-CGCCGTGGC TCCTGCTCT-3') and AK-VP-R1 (5'-TGTTCCGCAT-CACAAAAGATGTG-3') was used in the first PCR. Another pair of primers AK-VP-F2 (5'-GGCTCCT GCTCTAGGAAATAAAGAG-3') and AK-VP-R2 (5'-CCTGCTGTTAGGTCGTTGTTGTATGT-3') was used in the nested-PCR to amplify the partial VP1 gene of HBoV (576 basepairs). In addition, each sample was also screened for other respiratory viruses such as respiratory syncytial virus, influenza virus A and B, human metapneumovirus, parainfluenza virus type 1–4, human rhinoviruses, human coronaviruses, and adenovirus by using multiplex hemi-nested (RT)-PCR as described previously [Yoshida et al., 2010].

### Sequencing and Phylogenetic Analysis

All HBoV positive PCR products were sequenced by the commercial company (MacroGen Japan Corp., Tokyo, Japan). The nucleotide sequences were analyzed and compared with the reference strains available in the NCBI GenBank database. The sequence data and the phylogenesis were analyzed using BioEdit v.7.0.5 [Hall, 1999]. A parsimony analysis was also conducted using MEGA 5 [Tamura et al., 2011]. The method was performed using close-neighbor interchange with a random option and with 1,000 bootstrap repetitions.

The sequences of HBoV detected in this study have been deposited in the GenBank database under accession numbers JX418234–JX418266, KF193582–KF193604.

### Statistical Analysis

Categorical variables between groups were compared by using  $\chi^2$  test or Fisher's exact test, and continuous variables were compared by using the Mann–Whitney *U*-test. A two sided *P*-value  $<0.05$  was considered statistically significant. All analyses were conducted using SPSS 16.0 software (Chicago, IL).

## RESULTS

### Epidemiological Characteristics of HBoV Infection

Between April 2010 and May 2011, 1,082 children with acute respiratory infections were enrolled. The median age was 9 months (ranged from 1 to 161

months), 86% of patients were younger than 2 years old and the male to female ratio was 1.8:1. At least one respiratory virus was identified in 64.7% (700/1,082) of patients. Human rhinovirus and respiratory syncytial virus were the most common detected agents (30% and 23.8%, respectively). HBoV was found in 78 samples (7.2%). Among these children infected with HBoV, 52 (66.7%) were mixed infection with other respiratory viruses, most frequently with human rhinovirus ( $n=16$ ), followed by respiratory syncytial virus ( $n=15$ ). In addition, five cases had triple infection of HBoV, human rhinovirus and respiratory syncytial virus ( $n=3$ ), influenza A virus ( $n=1$ ), and parainfluenza virus type 3 ( $n=1$ ). The male to female ratio of HBoV positive patients was similar to that of the enrolled patients (1.7 vs. 1.8). The children infected with HBoV had a median age of 10 months (range from 1 to 62 months). Children younger than 6 months had the lowest infection rate (5.0%) while those aged from 12 to 23 months had the highest HBoV infection rate (9.8%; Fig. 1). HBoV was detected throughout the year. However, HBoV infections occurred more frequently during the dry season (December–April) than the rainy season (May–October; 9.8 vs. 5.7%,  $P$ -value = 0.015, Fisher's exact test; Fig. 2).

### Clinical Manifestations and Disease Severity

The clinical and demographic characteristics at presentation of all enrolled patients are shown in Table I. Patients with HBoV infection tended to be older than those negative for HBoV but the difference did not reach significance (median age 12 vs. 9 months). The difference on prematurity and malnutrition rates between HBoV-positive and HBoV-negative children was also not significant. Fever, cough, runny nose, wheezing, and rales were the most frequently observed clinical signs among HBoV-positive patients. Patients with HBoV infection had a significantly higher rate of hypoxia (16.7 vs. 7.9%,  $P$ -value = 0.007) which may result in longer hospitalized duration (median 6 vs. 5 days,  $P$ -value = 0.02) than those were negative for HBoV. Rales were present in 76.9% of HBoV-positive patients, significantly higher than that of HBoV-negative patients, 65.1% ( $P$ -value = 0.034). Vomiting and diarrhea were

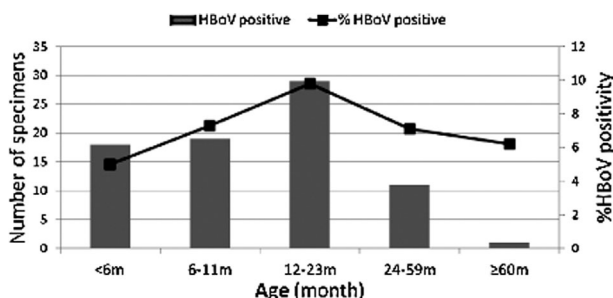


Fig. 1. Age distribution of HBoV infections among children with acute respiratory infection from April 2010 to May 2011.

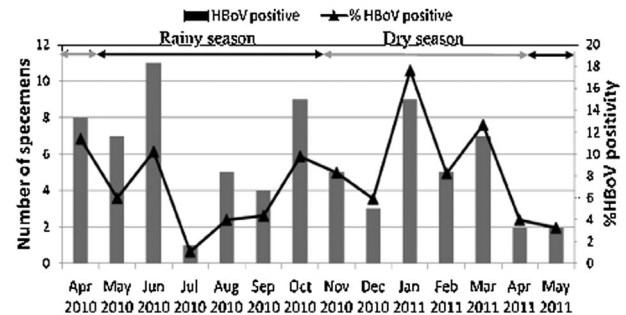


Fig. 2. Monthly distribution of HBoV infections from April 2010 to May 2011.

reported in 55.1% and 30.8% of patients with HBoV infection, respectively. Abnormal findings on chest X-ray were found in 82.1% of HBoV-positive patients. Regarding the diagnosis, HBoV was possible to cause diseases from the upper to the lower respiratory tract. In which, pneumonia was found more often in the children with HBoV infection than those without (50.0 vs. 38.2%,  $P$ -value = 0.04). No patient required mechanical ventilation. There was no fatal case.

Attempts were also made to compare the difference between HBoV mono- and co-infection groups (Table I). The median age of children with HBoV mono-infection was higher than those with co-infection (14.5 vs. 10.5 months). However, this difference was not statistically significant ( $P$ -value > 0.05). The demographic characteristics and clinical symptoms or signs in HBoV mono-infection and co-infection children were similar, except for prematurity, which was less common in HBoV mono-infection group (0 vs. 15.4%,  $P$ -value = 0.047, Fisher's exact test).

In the multivariate analysis adjusted for age, sex, prematurity, malnutrition, and infection with other viruses, the difference on the rate of hypoxia, rales and hospitalized duration remained significant between HBoV positive and negative group ( $P$ -value = 0.011, 0.037, and 0.030, respectively). However, the difference on pneumonia was no longer significant (OR: 1.59, 95% CI: 0.99–2.54,  $P$ -value = 0.053).

### Molecular Characterization and Phylogenetic Analysis

The partial VP1 nucleotide sequences (454 basepairs) of all 78 positive specimens for HBoV were determined and compared with those of four established HBoV species 1–4 and the prototype strains ST1 and ST2 (DQ000495 and DQ000496). The phylogenetic analysis (Fig. 3) showed that all HBoV strains isolated in this study were clustered closely in the same branch with HBoV1 reference strains. This finding confirmed that HBoV1 was circulating in Vietnam. The sequence identity of 97.5–100% at the nucleotide level and 94.6–100% at the amino acid level were observed among the HBoV positive strains isolated. All Vietnamese strains showed high nucleotide sequence identity of 97.5–100%

TABLE I. Demographic and Clinical Characteristics Associated With HBoV-Positive, Negative, Mono-, and Co-Infection Groups

Characteristics (%)	HBoV pos N = 78	HBoV neg N = 1,004	P-value	HBoV mono N = 26	HBoV co N = 52	P-value
Male	62.8	64.8	NS	65.4	61.5	NS
Age (month) <sup>a</sup>	12 (6–18)	9 (4–18)	NS	14.5 (9–17)	10.5 (5.5–19.5)	NS
Prematurity (<37 weeks)	10.3	9.1	NS	0	15.4	0.047 <sup>c</sup>
Malnutrition	12.8	9.3	NS	19.2	9.6	NS
Infection with other viruses	66.7	62.0	NS	NA	NA	
Days before hospitalization <sup>a</sup>	3 (2–4)	3 (2–5)	NS	3 (2–5)	2 (2–4)	NS
Fever	73.1	66.7	NS	65.4	76.9	NS
Cough	92.3	90.7	NS	92.3	92.3	NS
Runny nose	75.6	73.2	NS	80.8	73.1	NS
Vomiting	55.1	57.4	NS	46.2	59.6	NS
Diarrhea	30.8	27.0	NS	26.9	32.7	NS
SpO <sub>2</sub> ≤ 92%	16.7	7.9	0.007 <sup>b</sup>	11.5	19.2	NS
Tachypnea	46.2	44.4	NS	53.8	42.3	NS
Lower chest indrawing	55.1	55.2	NS	53.8	55.8	NS
Wheezing	62.8	58.6	NS	69.2	59.6	NS
Rales	76.9	65.1	0.034 <sup>b</sup>	73.1	78.8	NS
Abnormal chest X-ray	82.1	77.3	NS	80.8	82.7	NS
Diagnosis						
URTIs	23.1	21.5	NS	23.1	23.1	NS
Croup	3.8	6.3	NS	3.8	3.8	NS
Bronchiolitis	23.1	34.0	NS	19.2	25.0	NS
Pneumonia	50.0	38.2	0.04 <sup>b</sup>	53.8	48.1	NS
Hospitalization duration <sup>a</sup>	6 (4–9)	5(4–8)	0.02 <sup>d</sup>	6 (4–9)	6 (4.5–10)	NS

d, day; m, month; URTI, upper respiratory infection; HBoV, human bocavirus; NA, not applicable; NS, not significant; pos, positive; neg, negative; mono, mono-infection; co, co-infection.

All results are expressed in percentages except for (<sup>a</sup>) in median with interquartile range between brackets.

<sup>b</sup>Chi-squared test.

<sup>c</sup>Fisher's exact test.

<sup>d</sup>Mann-Whitney-U test.

and amino acid identity of 95.9–100% with HBoV1 reference strains. These sequences also shared 97.7–99.6% and 98.0–100% nucleotide sequence identity as well as 95.9–99.3% and 96.6–100% amino acid identity with the HBoV prototype strain ST1 and ST2, respectively. Interestingly, the partial VP1 sequences of three Vietnamese strains were identical to the HBoV prototype strain ST2.

## DISCUSSION

HBoV is a newly discovered human parvovirus that was first described in 2005 in nasopharyngeal aspirate of children with respiratory infection [Allander et al., 2005]. The discovery of this virus also raised the concerns about its causative role, as well as its community and clinical impact. Since then, HBoV has been detected frequently worldwide not only in the respiratory samples [Choi et al., 2006; Ma et al., 2006; Kesebir et al., 2006; Fry et al., 2007; Lau et al., 2007; Brieu et al., 2008; Yoshida et al., 2010] but also in human feces [Lau et al., 2007; Pham et al., 2011; Khamrin et al., 2012], serum [Allander et al., 2007; Christensen et al., 2010], saliva [Martin et al., 2009] and urine [Pozo et al., 2007]. The epidemiological status and genetic characteristics of HBoV circulating in children with acute respiratory infections in Vietnam, however, remains unknown. To understand better the epidemiology of HBoV infection, the presence of HBoV and other common

respiratory viruses in patients with acute respiratory infections in this region was investigated.

In this study, the incidence of HBoV infection was 7.2% among hospitalized children with acute respiratory infections. This rate is in accordance with previously published rates, ranging from 1.5% to 19% [Jartti et al., 2012]. The discrepancy observed in the incidence of HBoV infection between these studies may be explained by the differences in study design affecting age, recruitment criteria, study settings, study periods; the differences between hospital-based and community-based study; the differences in clinical specimen examination, diagnostic methods with different sensitivity and specificity, or regional and temporal differences. In this study, HBoV was the third most common respiratory agent after rhinovirus and respiratory syncytial virus, and it was more frequent than infections with influenza A, B, and parainfluenza viruses. Since the evidence of HBoV as a true respiratory pathogen becomes more convincing [Allander et al., 2005; Schildgen et al., 2008; Schildgen, 2010], the high detection rate implies that HBoV is responsible for a large burden of illness in young children with regard to health care utilization.

Regarding the season, although HBoV was detected throughout the year, its peak was in the dry season from December to April of this tropical area. However, no consistent seasonal distribution of HBoV was found in many previous reports. Most studies demonstrated that HBoV infection occurred year round with

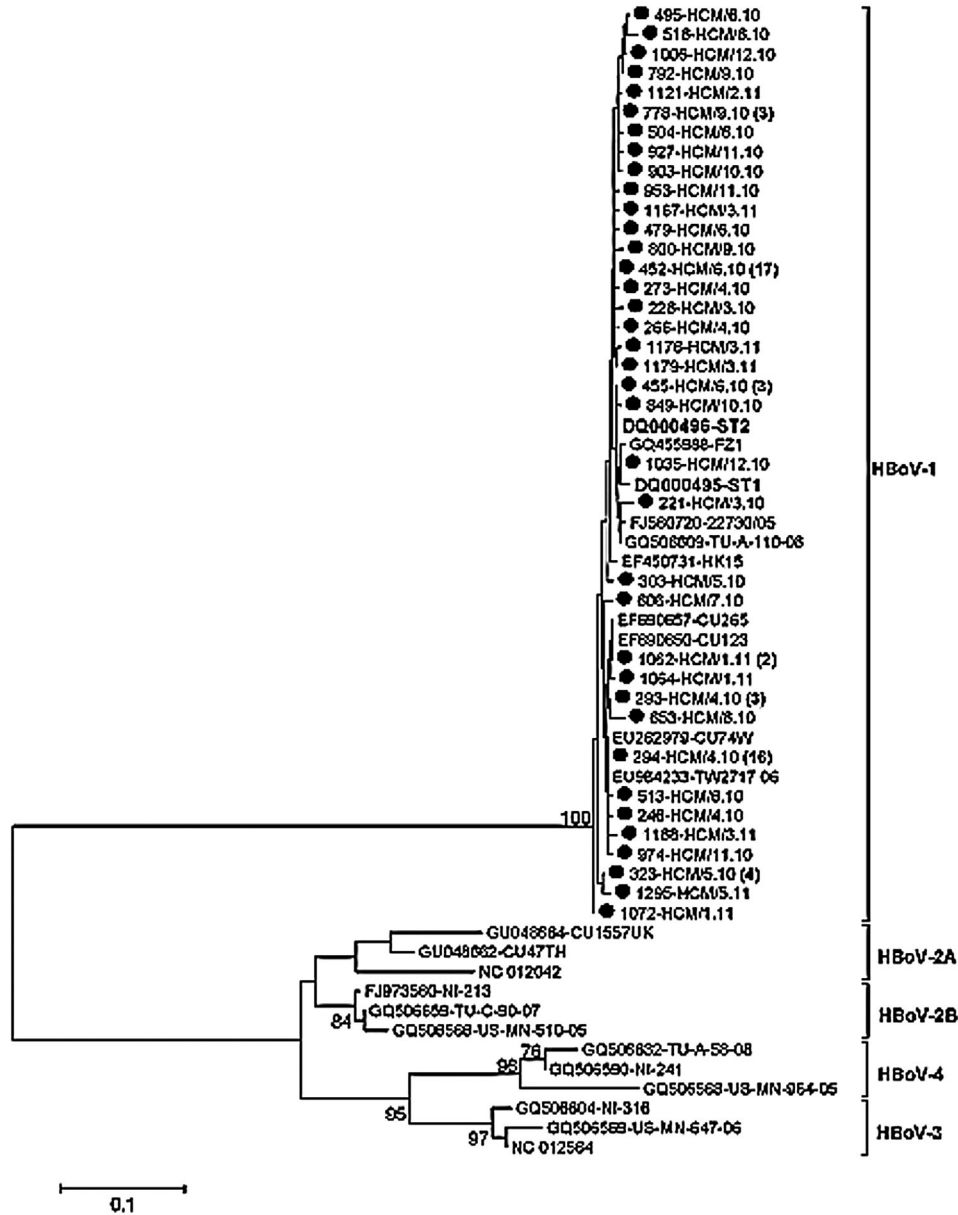


Fig. 3. Phylogenetic analysis of the partial VP1 nucleotide sequences of HBoVs. Phylogenetic tree was constructed with MEGA 5 software using the neighbor-joining method. Bootstrap values of greater than 70% are shown at the branch nodes. The HBoV strains in this study are marked with solid round. Prototype strain ST1 and ST2 (in bold face) were also included. Number of identical strains is indicated in the parentheses. The species assignment is indicated by the brackets on the right.

high prevalence in the winter and spring [Allander et al., 2005; Kesebir et al., 2006; Weissbrich et al., 2006; Lau et al., 2007; Pozo et al., 2007], while some other reports showed the increased HBoV infections in late spring and early summer [Choi et al., 2006; Ma et al., 2006]. On the other hand, some authors did not observe the obvious seasonal activity of HBoV [Bastien et al., 2006; Maggi et al., 2007; Zheng et al., 2010]. Since this study

spanned for about 1 year, in order to get a clear view on seasonality of HBoV infection, the continuing surveillance is necessary.

HBoV can be found in respiratory samples of all ages, but mainly in young children. In this study, the ages of HBoV-positive children ranged from 1 to 62 months, indicating that children over 5 years old were rarely infected with HBoV. The age group with the lowest infection rate was children younger than

6 months (5%). In contrast, the infection rate was highest among children 12–23 months (9.8%), the age at which most children have lost the maternal antibody protection, and decreased with the increasing age. These phenomena imply that maternal antibody against HBoV may protect the children during early infancy, and then, the immunity acquired from primary HBoV infection during early childhood may protect the children from getting later infection. The serological study in Japan revealed that nearly all individuals were seropositive for HBoV by the age of 5 years, and the seropositive rate was lowest in infants aged from 6 to 8 months and gradually increased with age [Endo et al., 2007].

The main symptoms of HBoV infection were cough, runny nose, fever and signs of respiratory distress such as tachypnea, lower chest retraction, wheezing, and hypoxia, which are also commonly seen among patients with respiratory infections. Therefore, it is impossible to differentiate clinically the respiratory infections caused by HBoV and other pathogens. However, in this study some clinical features, which were distinct for HBoV infection were also observed. Hypoxia was seen more often among children with HBoV-positive than those with HBoV-negative, which may lead to longer hospitalization duration. Moriyama et al. [2010] also observed that hypoxia was more severe in HBoV-positive patients than in RSV-positive ones. In this study, half of HBoV-positive patients were diagnosed as pneumonia. The existing literatures, in which HBoV infection was confirmed by serum PCR or serodiagnosis, also noted the significant association between HBoV infection and pneumonia [Fry et al., 2007; Christensen et al., 2010; Don et al., 2010; Karalar et al., 2010].

Co-detection of HBoV with other respiratory pathogens was found frequently, with the rate of up to 83% [Jartti et al., 2012]. However, HBoV viremia has been documented to be significantly more frequent in children with acute respiratory infections than in the control group, which supports the causative role of HBoV in respiratory diseases [Kesebir et al., 2006; Allander et al., 2007; Fry et al., 2007; Maggi et al., 2007]. In this study, 66.7% of children with HBoV-positive were co-infected with other viral pathogens. This result also confirmed that co-infection with other viruses was not an uncommon characteristic of HBoV. The overlapping seasonality of HBoV and other circulating viruses may explain for this phenomenon. Another explanation is the persistence, an interesting feature of HBoV [Jartti et al., 2012]. HBoV may persist in the airway rather long-lasting and the prolonged viral shedding may explain for the high co-detection rate observed in many studies. Unexpectedly, no correlation was found between co-infection and clinical symptoms. The rates of severe symptoms and lower respiratory tract infections did not increase in co-infection group. Co-infection of HBoV and other respiratory viruses did not increase the disease severity. A quantitative

PCR analysis may be helpful to clarify the role of HBoV to the respiratory diseases when being detected alone or together with other viruses.

The phylogenetic analysis of HBoV strains in this study and the reference strains revealed that HBoV species 1 was circulating in pediatric patients with acute respiratory infections in Vietnam. The recent review summarized the current knowledge on HBoV infection demonstrated that HBoV1 is predominantly found in respiratory samples, while the rest of HBoV species, including HBoV2, HBoV3, and HBoV4, have been found mainly in human stool [Jartti et al., 2012]. However, two studies of children with acute respiratory infections in Korea [Han et al., 2009] and China [Song et al., 2010] reported that the enteric HBoV2 was also identified in nasopharyngeal samples. Recently, the research from Japan reported the identification of all four HBoV species in respiratory samples from children with respiratory tract infections [Koseki et al., 2012]. To determine whether all HBoV species are involved with respiratory diseases, the pan-bocavirus primers were used in this study. However, only HBoV1 was found in children with acute respiratory infections in Vietnam and is in line with the above review's findings [Jartti et al., 2012]. The geographical differences may explain for the different distribution of HBoV species worldwide. The absence of HBoV2–4 in this study suggests these viruses may not directly involve in respiratory illnesses. The sequence analysis also revealed that the circulating HBoVs were closely related to the original strains. Most polymorphisms identified in this study did not result in amino acid changes at the corresponding protein. The high similarity suggests that HBoV1 genes may be highly conserved, and serological tests should be universal application and interpretation worldwide.

In conclusion, this research provides the first data on the molecular background of HBoV among children with respiratory infections in Vietnam. Information from this study will contribute to the growing database on the molecular diversity of HBoV circulating worldwide. Moreover, these data indicated that HBoV seems to be an important and frequent pathogen in respiratory tract infection in children. Further surveillance and molecular characterization of HBoV including the healthy control group and using serology and/or PCR detection of HBoV in blood is essential to clarify the clinical impact as well as to provide further genetic information of HBoV.

## ACKNOWLEDGMENTS

We deeply thank doctors and nurses, especially Ms. Pham Thi Ngoc Hue, Children's Hospital 2, Ho Chi Minh City for their help in patient counseling and sample collection. DNT is a recipient of the scholarship from the Honjo International Scholarship Foundation.

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