

Clinical and Molecular Epidemiology of Human Bocavirus in Respiratory and Fecal Samples from Children in Hong Kong

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(See the editorial commentary by Mackay, on pages 968–70.)

Background. Human bocavirus (HBoV) is a recently discovered parvovirus associated with respiratory tract infections in children. We conducted the first systematic prospective clinical and molecular study using nasopharyngeal aspirates (NPAs) and fecal samples.

Methods. NPAs negative for influenza virus, parainfluenza virus, respiratory syncytial virus, adenovirus, and coronavirus and fecal samples from patients with acute gastroenteritis were included. On the basis of results from a pilot study using 400 NPAs from all age groups, a prospective 12-month study was conducted to detect HBoV in 1200 NPAs and 1435 fecal samples from patients <18 years old by polymerase chain reaction. The complete genome sequences of HBoVs from 12 NPAs and 12 fecal samples were determined.

Results. Of the 400 NPAs collected in the pilot study, 20 (5.0%) were found to contain HBoV, all from children <5 years old. In the subsequent prospective study of pediatric patients, HBoV was detected in 83 (6.9%) of 1200 NPAs. Upper and lower respiratory tract infections were equally common. HBoV was detected in 30 (2.1%) of 1435 fecal samples. Fever and watery diarrhea were the most common symptoms. The seasonality of HBoV in NPAs and fecal samples was similar. Codetection with other pathogens occurred in 33% and 56% of NPAs and fecal samples, respectively, from patients with HBoV infection. Genomes of HBoVs from NPAs and fecal samples displayed minimal sequence variations.

Conclusions. HBoV was detected in fecal specimens in children with acute gastroenteritis. A single lineage of HBoV was associated with both respiratory tract and enteric infections.

Because a substantial proportion of respiratory tract infections remain undiagnosed [1, 2], research has been conducted to identify novel causative agents. Over the past few years, several novel respiratory viruses—including human metapneumovirus (hMPV) [3], severe acute respiratory syndrome (SARS) coronavirus (SARS-

CoV) [4], human coronavirus NL63 (HCoV-NL63) [5, 6], and coronavirus HKU1 (CoV-HKU1) [7–10]—have been identified.

In 2005, Allander et al. [11] reported the discovery of a previously undescribed human parvovirus in respiratory secretions from children with respiratory tract disease in Sweden. Phylogenetic analysis showed that this virus belonged to the genus *Bocavirus* (subfamily, Parvovirinae; family, Parvoviridae) and was most closely related to bovine parvovirus (BP) and minute virus of canines (MVC). The virus was thus named

Received 4 March 2007; accepted 19 March 2007; electronically published 29 August 2007.

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The Journal of Infectious Diseases 2007;196:986–93

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0022-1899/2007/19607-0007\$15.00

DOI: 10.1086/521310

Potential conflicts of interest: none reported.

Financial support: Research Grant Council; University of Hong Kong (University Development Fund and Outstanding Young Researcher Award); Tung Wah Group of Hospitals Fund for Research in Infectious Diseases; Hong Kong Special Administrative Region Research Fund for the Control of Infectious Diseases of the Health, Welfare, and Food Bureau.

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“human bocavirus” (HBoV). By a specific polymerase chain reaction (PCR) assay, the virus was found to be present in 17 (3.1%) of 540 respiratory specimens collected from hospitalized children over a 1-year period [11].

Subsequently, HBoV has been reported in respiratory samples from children in various parts of the world (including Australia, North America, Europe, Asia, and Africa), suggesting that the virus is circulating worldwide [12–24]. Among these studies, HBoV was detected in 1.5%–18.3% of respiratory samples from individuals with acute respiratory tract illness, especially those from young children and infants. Despite these findings, the clinical spectrum of disease and a causative role for this novel virus remain to be ascertained. In a recent study, HBoV DNA was found to be frequently present in serum samples from patients with acute wheezing, suggesting that the virus may be associated with systemic infection [25]. However, it is not known whether HBoV is associated with nonrespiratory tract illness and can be detected in other clinical specimens. Moreover, few studies to date have included specimens from older children or adults.

To assess the epidemiology of HBoV infection in our population, in a pilot study we examined the prevalence of HBoV in randomly selected nasopharyngeal aspirates (NPAs) from hospitalized patients of all ages. On the basis of the results of this pilot study, a 1-year prospective study was conducted using NPAs and fecal samples from pediatric patients to determine clinical disease associations and the epidemiology of HBoV infection in children in Hong Kong. The molecular epidemiology of HBoV detected in NPAs and fecal samples was also analyzed.

METHODS

Patients and microbiological methods. All samples were collected from hospitalized patients at 3 hospitals in Hong Kong. All NPAs were tested for influenza viruses A and B; parainfluenza viruses 1, 2, and 3; respiratory syncytial virus; and adenovirus by direct immunofluorescence. NPAs were also tested for human coronavirus 229E, human coronavirus OC43, HCoV-NL63, and CoV-HKU1 by reverse-transcription PCR (RT-PCR) [9, 10, 26]. As a pilot study to determine the age distribution of HBoV infection in our population, 200 NPAs negative for the respiratory viruses listed above (118 from pediatric patients [<18 years old] and 92 from adult patients [≥ 18 years old]) and 200 NPAs positive for any of these viruses (166 from pediatric patients and 34 from adult patients) sent to the microbiology laboratories were randomly selected for HBoV testing by PCR.

Because all positive samples from the pilot study were from young children, a prospective study that aimed to determine the epidemiology of HBoV infection and the clinical spectrum of disease caused by this infection was done using specimens

from pediatric patients during a 12-month period (November 2004–October 2005). One hundred NPAs from pediatric patients that tested negative for the respiratory viruses listed above were randomly selected each month for HBoV testing by PCR. During the same period, fecal samples were also collected from pediatric patients with acute gastroenteritis, which was defined as the development of acute diarrhea with 3 or more loose stools per day. All fecal samples were tested for common bacterial diarrheal pathogens, rotavirus (by antigen detection [27]), and HBoV (by PCR). When HBoV was detected, the corresponding patients were identified, and their clinical features, laboratory results, and outcome were analyzed retrospectively.

PCR for HBoV and sequencing. DNA from NPAs and fecal samples was extracted using the QIAamp DNA Mini Kit (Qiagen), in accordance with the manufacturer’s protocol. DNA was subjected to PCR for HBoV as described elsewhere, using forward primer 5′-GAGCTCTGTAAGTACTATTAC-3′ and reverse primer 5′-CTCTGTGTTGACTGAATACAG-3′ targeted to a 354-bp fragment of the *NP1* gene; the primers were based on the corrected sequences published as a corrigendum [11]. The amplified products were detected by agarose gel electrophoresis. Both strands of all PCR products were sequenced twice with an ABI Prism 3700 DNA Analyzer (Applied Biosystems), using the PCR primers. The sequences of the PCR products were compared with the sequences of HBoV strains available in GenBank.

Detection of hMPV and rhinovirus by RT-PCR. There was no significant difference in the rate of detection of HBoV between NPAs that were positive and those that were negative for other respiratory viruses, which raised the possibility that concomitant respiratory viruses that were not included in our tests may also be present in the NPAs. Therefore, stored RNA from all NPAs positive for HBoV was retrieved and subjected to RT-PCR for hMPV and rhinovirus. Viral RNA was extracted from NPAs by use of the QIAamp Viral RNA Mini Kit (Qiagen). RT was performed using random hexamers and the SuperScript II Kit (Invitrogen) [7, 8]. PCR for hMPV and rhinoviruses was performed using protocols described elsewhere [28, 29].

Complete genome sequencing and phylogenetic analysis of the NS1, NP1, and VP1/VP2 genes of HBoV. The complete genomes of HBoVs from 12 NPAs and 12 fecal samples were amplified and sequenced using a strategy that has been described elsewhere [8]. Primers were designed by multiple alignment of the genomes of HBoV available in GenBank, and additional primers were designed on the basis of the results of the first and subsequent rounds of sequencing. The terminal sequences were confirmed by a modified protocol for rapid amplification of cDNA ends [11]. However, these terminal sequences may be incomplete because of their hairpin structures. The nucleotide and the deduced amino acid sequences of the *NS1*, *NP1*, and *VP1/VP2* genes were compared with those of

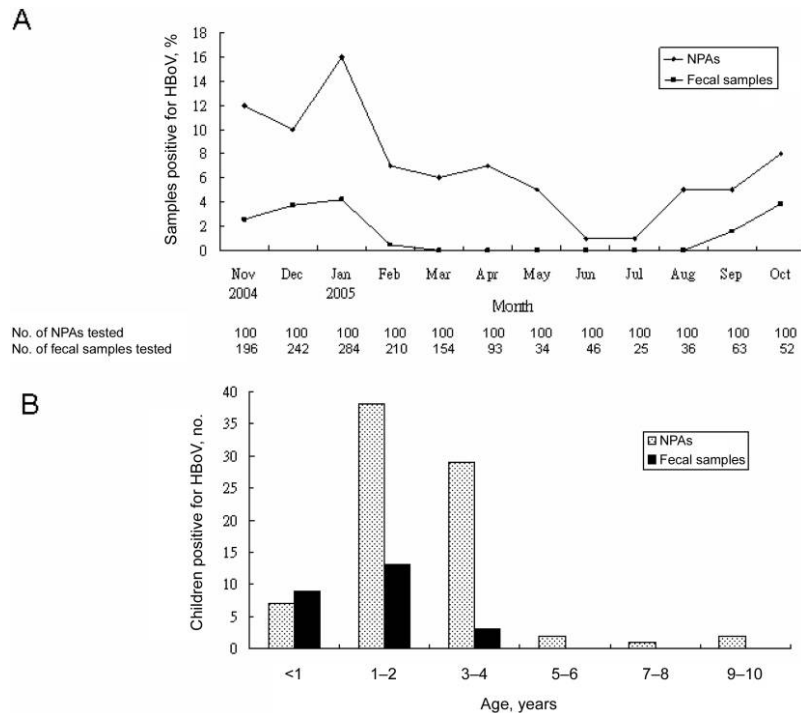


Figure 1. Seasonality of human bocavirus (HBoV) in nasopharyngeal aspirates (NPAs) and fecal samples (A) and distribution of HBoV-positive children by age (B).

HBoV strains with a complete genome sequence available in GenBank. Phylogenetic trees were constructed by the neighbor-joining method with GrowTree, using Kimura's 2-parameter correction with ClustalX 1.83 (Genetics Computer Group).

Nucleotide sequence accession numbers. The complete genome sequences of the 24 strains of HBoV identified here have been deposited in GenBank under accession numbers EF450717–EF450740.

RESULTS

Detection of HBoV in NPAs from pediatric patients. Of the 200 NPAs that were negative for influenza A and B viruses; parainfluenza viruses types 1, 2, and 3; respiratory syncytial virus; adenovirus; and the 4 human coronaviruses, 7 (3.5%) were positive for HBoV. Of the 200 NPAs that were positive for any of these respiratory viruses, 13 (6.5%) were positive for HBoV. All of the 20 HBoV-positive NPAs were from children <5 years old. The detection rate in pediatric patients (<18 years old; 20/284 [7%]) was significantly higher than that in adults (≥ 18 years old; 0/126 [0%]) ($P < .005$, χ^2 test). Therefore, the subsequent prospective study was conducted in pediatric patients.

During the 12-month prospective study period, 1200 NPAs (100 per month) from pediatric patients (male to female ratio, 1.6:1; mean \pm SD age, 3.8 ± 3.9 years) were subjected to PCR for HBoV. HBoV was detected in 83 NPAs. These 83 NPAs

were obtained from 79 patients who were <10 years old (median age, 2 years; range, 6 months–9 years). Since 1081 of the 1200 NPAs were obtained from patients <10 years old, the 83 positive NPAs represent a 7.7% (83/1081) detection rate in this population. Of the 79 patients positive for HBoV, 46 were male, and 33 were female. HBoV was detected throughout the year, with the highest prevalence during fall and winter (figure 1).

Of the 79 patients, 36 had received a clinical diagnosis of upper respiratory tract infection (URTI), and 35 had a lower respiratory tract infection (LRTI). The remaining 8 patients did not have respiratory symptoms (table 1). Of the 35 patients with LRTI, 27 had pneumonia, 6 had acute bronchitis/bronchiolitis, and 2 had croup. One patient with pneumonia had *Streptococcus pneumoniae* isolated from the sputum. Although fever, cough, and rhinorrhea were the most common symptoms in patients with URTI, 2 had acute pharyngitis (with β -hemolytic group A streptococcus isolated from a throat swab in one of them), and 1 patient had acute sinusitis. Three patients with URTI and 3 with LRTI also had acute nonsuppurative otitis media. Fourteen patients experienced an asthma exacerbation, with 1 complicated by status asthmaticus. A number of patients with acute respiratory illness also had nonrespiratory symptoms or additional diagnoses. Nine had febrile convulsion, and 2 others with underlying epilepsy had breakthrough seizures. Six had symptoms of gastroenteritis, and 1 had intussusception. One had pneumonia and gastroenteritis compli-

Table 1. Clinical characteristics of the patients with human bocavirus (HBoV) detected in nasopharyngeal aspirates.

Characteristic	Value
Patients, total no.	79
Male to female ratio	46:33
Age, median (range)	2 years (6 months–9 years)
Diagnosis ^a	
URTI	36 (46)
Acute pharyngitis	2 (3)
Acute sinusitis	1 (1)
Acute otitis media	6 (8)
Acute bronchitis/bronchiolitis	6 (8)
Pneumonia	27 (34)
Croup	2 (3)
Asthma exacerbation	14 (18)
Febrile convulsion	9 (11)
Breakthrough seizure	2 (3)
Aseptic meningitis	1 (1)
Gastroenteritis	9 (11)
Acute hepatitis	1 (1)
Intussusception	1 (1)
Kawasaki disease	2 (3)
Infectious mononucleosis	2 (3)
Herpangina	1 (1)
Herpetic gingivostomatitis	2 (3)
Henoch-Schönlein purpura	1 (1)
Roseola infantum	1 (1)
Nephrotic syndrome	1 (1)
Copathogens	
<i>Streptococcus pyogenes</i>	1 (1)
<i>Streptococcus pneumoniae</i>	1 (1)
Enterovirus	2 (3)
Epstein-Barr virus	2 (3)
Rotavirus	2 (3)
Herpes simplex virus	2 (3)
Rhinovirus	14 (18)
Human metapneumovirus	2 (3)

NOTE. Data are no. (%) of patients, unless otherwise indicated. URTI, upper respiratory tract infection.

^a Percentages sum to >100% because some patients had >1 diagnosis.

cated by nephrotic syndrome. Two others with pneumonia had primary Epstein-Barr virus infection. One patient with URTI had herpangina, 2 had herpetic gingivostomatitis, and another had Henoch-Schönlein purpura. Of the 8 patients without respiratory symptoms, 3 had gastroenteritis, 2 of whom had rotavirus antigen detected in their fecal samples. Two had Kawasaki disease. One had aseptic meningitis due to enterovirus. One had acute hepatitis of unknown etiology. One had roseola infantum. Interestingly, HBoV was persistently detected in a 2-year-old boy in 5 separate NPAs collected over a 1-month period. Apart from the HBoV infection in this patient, who had

URTI complicated by recurrent wheezing during his prolonged hospitalization for short gut syndrome, all cases were community acquired. All 79 patients survived.

Detection of HBoV in fecal samples from pediatric patients with acute gastroenteritis. During the 12-month period, 1435 fecal samples from pediatric patients with acute gastroenteritis (male to female ratio, 1.6:1; mean \pm SD age, 2.7 \pm 3.4 years) were subjected to PCR for HBoV. HBoV was detected in 30 samples (2.1%) from 25 patients (tables 2 and 3). The median age of these patients was 17 months (range, 2 months–3 years). Eighteen were male and seven were female. As with the results for NPAs, HBoV was mainly detected in fecal samples during the fall and winter months, with all cases occurring from September to February. Diarrhea lasted for 1–4 days, and the frequency of stool passage ranged from 3–20 times per day. Blood was present in the stool of 4 patients and in the mucus of 2. Eight experienced vomiting, and 17 had fever. Coryzal symptoms occurred in 14 patients. Seven had LRTI, with pneumonia in 3 and acute bronchiolitis in 4. One also had urinary tract infection. Two had febrile convulsions, and 1 with underlying epilepsy had recurrent seizures. Diarrheal pathogens were frequently found in fecal samples, with rotavirus identified in 9, serogroup B *Salmonella* species in 2, *Campylobacter* species in 1, and *Staphylococcus aureus* in 1. HBoV was repeatedly detected in separate fecal samples from 3 patients. In particular, HBoV

Table 2. Summary of characteristics of the patients with human bocavirus detected in fecal samples.

Characteristic	Value
Patients, total no.	25
Male to female ratio	18:7
Age, median (range)	17 months (2 months–3 years)
Clinical manifestations/other diagnoses ^a	
Presence of blood in stool	4 (16)
Presence of mucus in stool	2 (8)
Vomiting	8 (32)
Fever	17 (68)
Coryzal symptoms	14 (56)
Acute bronchiolitis	4 (16)
Pneumonia	3 (12)
Febrile convulsion	2 (8)
Breakthrough seizure	1 (4)
Urinary tract infection	1 (4)
Copathogens	
Rotavirus	9 (36)
<i>Salmonella</i> species	2 (8)
<i>Campylobacter</i> species	1 (4)
<i>Staphylococcus aureus</i>	1 (4)
<i>Clostridium difficile</i>	1 (4)

NOTE. Data are no. (%) of patients, unless otherwise indicated.

^a Percentages sum to >100% because some patients had >1 diagnosis.

Table 3. Clinical characteristics of the 25 patients with human bocavirus detected in fecal samples.

Patient	Month of detection	Sex	Age	Underlying disease	Diagnosis other than gastroenteritis	Codetection in fecal sample	Multiple detection (days apart)
1	Nov	Male	2 years	Autism	None	None	ND
2	Nov	Male	16 months	None	None	Rotavirus	ND
3	Nov	Male	2 months	None	Acute bronchiolitis	None	ND
4	Nov	Male	13 months	None	Pneumonia	None	ND
5	Nov	Male	3 years	None	None	<i>Campylobacter</i>	ND
6	Dec	Male	7 months	Sacral mass	None	None	ND
7	Dec	Female	19 months	None	Pneumonia	None	ND
8	Dec	Male	20 months	Febrile convulsion	Febrile convulsion	Rotavirus	ND
9	Dec	Male	4 months	History of urinary tract infection	Urinary tract infection	None	ND
10	Dec	Male	8 months	None	Acute bronchiolitis	None	ND
11	Dec	Male	16 months	None	None	Rotavirus	ND
12	Dec	Female	16 months	Epilepsy	Breakthrough seizures	Rotavirus	ND
13	Dec	Male	5 months	History of urinary tract infection	None	Serogroup B <i>Salmonella</i>	ND
14	Dec	Male	20 months	None	None	Rotavirus	ND
15	Jan	Female	9 months	Ring chromosome 18, congenital heart disease, hydrocephalus	None	None	ND
16	Jan	Female	3 years	Nephrotic syndrome	Pneumonia	Rotavirus	Yes (3)
17	Jan	Male	3 years	Smith-Lemli-Opitz syndrome, myelodysplastic syndrome	Acute bronchiolitis	<i>Clostridium difficile</i>	Yes (35)
18	Jan	Male	18 months	Febrile convulsion	Febrile convulsion	None	ND
19	Jan	Male	3 months	None	None	Rotavirus, <i>Staphylococcus aureus</i>	ND
20	Jan	Female	2 years	Febrile convulsion	None	Rotavirus	ND
21	Jan	Male	2 years	Short gut syndrome, chronic lung disease	None	None	Yes (2)
22	Jan	Male	7 months	None	Acute bronchiolitis	Rotavirus	ND
23	Sep	Male	18 months	None	None	Serogroup B <i>Salmonella</i>	ND
24	Oct	Female	19 months	B cell immunodeficiency	None	None	ND
25	Oct	Female	5 months	None	None	None	ND

NOTE. ND, not done.

was persistently found in 4 samples collected over 5 weeks from a 3-year-old boy (patient 17 in table 3) with multiple congenital malformations who developed persistent diarrhea due to *Clostridium difficile* during hospitalization. Apart from the HBoV infection in this patient, all cases were community acquired. All patients survived.

Detection of hMPV and rhinovirus in NPAs. Available RNA from 93 NPAs positive for HBoV was subject to RT-PCR for hMPV and rhinovirus. Of these 93 samples, 14 were positive for rhinovirus, and 2 were positive for hMPV. Rhinovirus was detected in 6 with LRTI and 8 with URTI. Ten of these 14 patients with rhinovirus infection had asthma exacerbations, and 2 had febrile convulsions. hMPV was detected in 1 patient with LRTI and in 1 patient with URTI and gastroenteritis.

Complete genome sequencing and phylogenetic analysis of the NS1, NP1, and VP1/VP2 genes of HBoV. The complete genomes of HBoVs from 12 NPAs and 12 fecal samples were amplified and sequenced. The 24 HBoV genomes (size, 5.2 kb) had G+C content of 42%. The genomic organization was the same as that of the 2 prototype HBoV strains, ST1 and ST2, and of other known bocaviruses, BPV and MVC. There were

3 open reading frames (ORFs), with the gene order *NS1*, *NP1*, and *VP1/VP2*. Sequence analysis showed that the 24 strains of HBoV displayed limited sequence variations among themselves as well as in relation to the available sequences of previously described HBoV strains in all 3 genes (figure 2). The *NS1* gene was the most conserved gene among the 3, with only minor nucleotide polymorphisms. The predicted amino acid sequences of the *NS1* gene of the 24 HBoV strains were identical among themselves and in relation to *NS1* sequences of HBoVs from GenBank, except for a strain from China (HBoV_WLL-1) that had 1 amino acid substitution (His→Arg) resulting from a mutation at nucleotide position 1328. The greatest sequence variations occurred in the *VP1/VP2* gene, although the overall nucleotide and amino acid differences among different strains were <2% and <1%, respectively.

DISCUSSION

Although the results of this study of NPAs are similar to those of previous studies, the present report is the first to document the presence of HBoV in fecal specimens. In this 1-year pro-

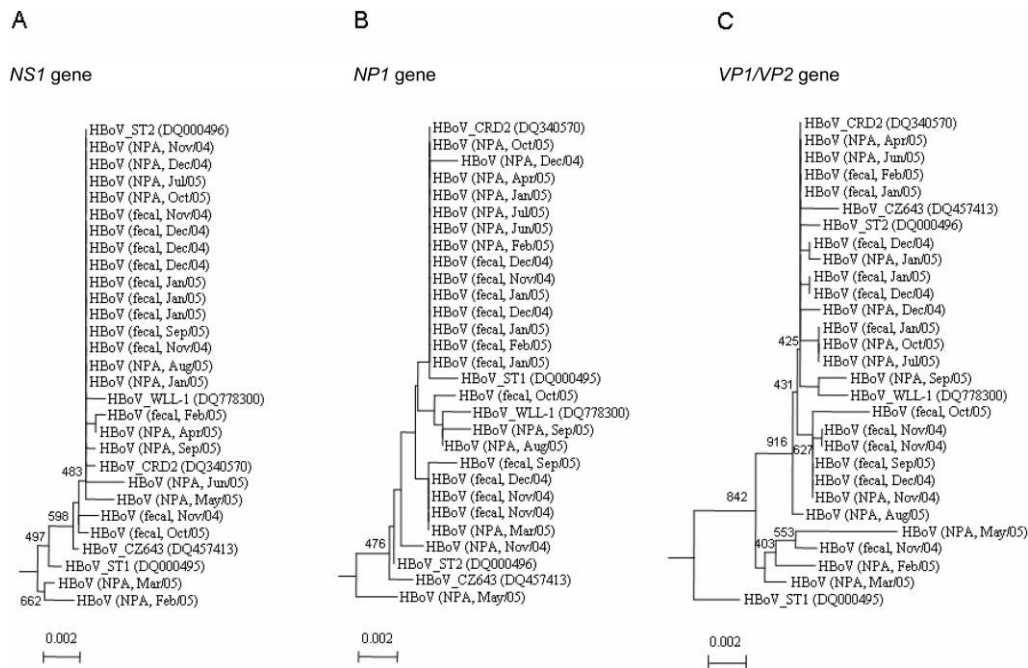


Figure 2. Phylogenetic trees of complete *NS1*, *NP1*, and *VP1/VP2* gene sequences of 12 human bocavirus (HBoV) strains from nasopharyngeal aspirates (NPAs) and 12 HBoV strains from fecal samples. The trees were inferred from *NS1* (A), *NP1* (B), and *VP1/VP2* (C) gene data by the neighbor-joining method, using bootstrap values calculated from 1000 trees. The trees were rooted using the *NS1*, *NP1*, and *VP1/VP2* gene sequences of minute virus of canine; 1920 nucleotide positions in each *NS1* gene, 660 nucleotide positions in each *NP1* gene, and 2016 nucleotide positions in each *VP1/VP2* gene were included in the analysis. The scale bar indicates the estimated no. of substitutions per 500 bases by Kimura's 2-parameter model. HBoV strains ST1 and ST2, from Sweden, were the 2 prototype strains. Strains WLL-1 and CZ643 are from China, and strain CRD2 is from the United States.

spective study, HBoV was found in NPAs from 79 children, 71 of whom had acute respiratory tract illness. Similar to previous studies, most of our patients with HBoV infection were infants or young children. Although on occasion adults have been reported to be infected with HBoV [22, 30], none of the NPAs from adults in the present study was positive for HBoV. Among patients with acute respiratory tract illness, URTI and LRTI were equally common. Although asthma exacerbations, febrile convulsions, and gastroenteritis were common associated clinical manifestations, other apparently unrelated diagnoses were also frequent. Among those patients without respiratory symptoms but with HBoV detected in NPAs, gastroenteritis was the most common diagnosis, in agreement with the detection of HBoV in fecal samples from children with gastroenteritis. In a previous study in the United States, it was found that 5 HBoV-positive children had diarrhea [14]. However, it was not known whether HBoV could be detected in their fecal samples. In the present study, HBoV was detected in 2.1% of fecal samples from children with gastroenteritis. Fever and watery diarrhea were the most common manifestations, followed by coryzal symptoms and vomiting. Similar to findings for NPAs, the affected children were young (≤ 3 years of age). The seasonal epidemiological profile of HBoV in fecal samples was similar

to that in NPAs, with the highest incidence in fall and winter. The finding of HBoV in both respiratory and enteric specimens from humans concurs with the characteristics of the other 2 members of the genus *Bocavirus*, BP and MVC. BP causes diarrhea and mild respiratory symptoms in calves [31, 32]. Although MVC is associated with fetal infections and neonatal respiratory disease in dogs, the virus has occasionally been associated with enteritis [33, 34]. Further studies are required to determine the role played by HBoV in enteric disease in humans.

Although there is no doubt that HBoV is a common human virus acquired early during life, the causative role of HBoV in human disease remains to be determined. Despite the large number of studies of HBoV conducted, it was not until recently that a control group of asymptomatic children was included. In this study from the United States, HBoV was identified in 22 (5.2%) of 425 samples from symptomatic children but in none from the asymptomatic control children ($P = .02$) [14], supporting an association between HBoV and respiratory tract illness. However, although respiratory specimens from their symptomatic children included nasal swabs, respiratory secretions, and bronchoalveolar lavage fluid sent to a hospital-based laboratory, only nasal wash specimens were obtained from

asymptomatic control children at a primary care center. Because it was not indicated whether any of the HBoV-positive specimens were nasal washes, it is difficult to conclude whether the observed difference was genuine or due to discrepancies in sampling. Nevertheless, in another study, HBoV also appeared to be more common among individuals with respiratory tract disease than among those without symptoms [15]. Although the association between HBoV and respiratory tract disease may support a role for the virus in pathogenesis, it does not prove causality, which is often difficult to ascertain in the case of respiratory viruses [35].

Although a lack of proof of causality by Koch's postulates does not exclude the possibility that HBoV is a pathogen, its exceptionally high frequency of codetection with other respiratory viruses, a phenomenon that was not observed for known respiratory viruses, has led to reservations concerning its role in human disease. The frequency of codetection in HBoV-positive respiratory samples has ranged from 33% to 80%, depending on how intensively other respiratory viruses were sought, the sensitivities of the methods used, and the sampling size [12, 16–21]. The highest codetection rate—observed in a Swiss study in which 4 of 5 HBoV-positive nasal samples from infants contained other respiratory viruses—may have been due to the inclusion of a comprehensive panel of respiratory viruses that included rhinovirus and coronaviruses [16]. In the present study, although many common respiratory viruses were initially excluded from the NPAs selected for HBoV testing, 33% of the patients with NPAs positive for HBoV were subsequently found to have coinfections with another pathogen. As for fecal samples, a high rate of coinfection (56%) was also observed. It has been reported that the frequency of HBoV infection was even higher when another virus was present [15]. In the present study, a higher prevalence was also observed in NPAs positive for common respiratory viruses (6.5%) than in those that were negative (3.5%). Further studies are required to determine whether HBoV plays a causative role in these coinfections or acts as an exacerbating factor that simply increases the severity of infections caused by other pathogens.

The low genetic diversity of HBoV suggested that a single lineage was responsible for both respiratory tract and enteric infections in humans. This is in contrast to coronaviruses, which have different genotypes, possibly as a result of a high frequency of recombination [36]. The 5.2-kb genome of HBoV contains 3 ORFs encoding NS1 (a nonstructural protein), NP1 (a protein of unknown function), and VP1/VP2 (viral capsid protein). Phylogenetic analysis of these 3 ORFs in all 24 HBoV strains from NPAs and fecal samples did not reveal a genotypic difference between the strains from NPAs and those from fecal samples. Among the 3 genes, *NS1* appeared to be the most conserved. Similar to the findings of previous studies, which mostly examined the partial *NP1* gene, minor nucleotide sub-

stitutions were observed in *NP1*, with occasional amino acid substitutions. When HBoV was first described, it was found that most nucleotide polymorphisms occurred in *VP1/VP2* [11]. Only 2 subsequent studies have analyzed partial sequences of *VP1/VP2*. In one study, which involved the sequencing of a 1-kb fragment of the *VP2* gene, 97.5%–100% nucleotide sequence identity was observed between their HBoV strains and the prototype strains, suggesting a unique lineage of HBoV [18]. In another study, which analyzed the 3' 819-bp fragment of *VP1/VP2*, the authors suggested that 2 distinct genotypes were observed, with ST1 being in one cluster and ST2 in another [14]. In the present study, comparison of the complete *VP1/VP2* gene sequences from our 24 HBoV strains and strains from other countries showed only minor variations. Although phylogenetic analysis of this gene showed that there may be clustering among some strains, the existence of distinct genotypes cannot be concluded. In fact, our present results suggest that humans are infected by a single lineage of HBoV, which was detected in both respiratory tract and enteric samples.

The lack of variation in the surface protein of HBoV suggests that HBoV infection may happen only once, with the subsequent development of life-long immunity via neutralizing antibody. This is consistent with the fact that HBoV infection occurs primarily in infants and young children. The development of immunity against HBoV and the clearance of HBoV may depend on the integrity of one's immune system. In the Swiss study [16], HBoV was detected in follow-up samples from only 1 of the 5 infants, suggesting that HBoV is rapidly cleared in most cases. In the present study, persistent HBoV shedding for >1 month was observed in both respiratory tract and fecal specimens from patients with significant underlying diseases, which may represent prolonged infections as a result of underlying immunosuppression. However, routine follow-up was not done in our patients, because our clinical data were mainly collected retrospectively after HBoV PCR results had become available, by which time most patients had been discharged. Larger longitudinal studies should be conducted to examine the duration of HBoV shedding and the mechanism of immunity.

Acknowledgments

We are grateful for the generous support of Mr. Hui Hoy and Mr. Hui Ming with respect to the genomic platform.

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