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Human Bocavirus Infection in Young Children With Acute Respiratory Tract Infection in Lanzhou, China

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Human bocavirus (HBoV) is a recognized human parvovirus associated with acute respiratory tract infection. However, HBoV has yet to be established as a causative agent of respiratory disease. In this study, the epidemiological and virological characteristics of HBoV infection were studied in children with acute respiratory tract infection in China. In total, 406 children younger than 14 years of age with acute respiratory tract infection were included in this prospective 1-year study. HBoV was detected in 29 (7.1%) of the 406 children. No clear seasonal fluctuation was observed in infection rates of HBoV. Of the 29 children infected with HBoV, 16 (55.2%) were coinfected with other respiratory viruses, most commonly respiratory syncytial virus (RSV). Viral coinfection with HBoV did not affect the severity of the respiratory disease ($P = 0.291$). The number of HBoV genome copies ranged from 5.80×10^2 to 9.72×10^8 copcopies/ml in nasopharyngeal aspirates among HBoV-positive specimens by real-time PCR, and neither coinfection nor the severity of disease correlated with the viral load ($P = 0.148$, $P = 0.354$, respectively). The most common clinical features were cough and acute upper respiratory infection, and acute bronchopneumonia. Additionally, the NP-1 gene of HBoV showed minimal sequence variation. These data suggest that HBoV is frequent in young children with acute respiratory tract infection in Lanzhou, China, and RSV is the most common coinfecting virus. There was no apparent association between the viral load of HBoV and coinfection or disease severity. The NP-1 gene was highly conserved in HBoV. ***J. Med. Virol. 82:282–288, 2010.***

KEY WORDS: human bocavirus; acute respiratory tract infection; coinfection; children

INTRODUCTION

Human bocavirus (HBoV) is a human parvovirus which was identified in 2005 in clinical specimens from infants and children suffering from respiratory tract illness. Phylogenetic analyses of the complete genome of HBoV revealed that the virus is related most closely to canine minute virus and bovine parvovirus, which are members of the *Bocavirus* genus of the *Parvoviridae* family [Allander et al., 2007]. The worldwide distribution of HBoV was demonstrated subsequently by detection of the virus in patients with acute respiratory infection in many countries. The relative importance of HBoV as a causative agent for viral respiratory illnesses has not yet been determined, but it has been associated with respiratory illnesses ranging from upper respiratory tract disease to severe bronchiolitis and pneumonia [Ma et al., 2006; Weissbrich et al., 2006; Fry et al., 2007; Fabbiani et al., 2009; Tan et al., 2009]. Although HBoV has been detected in patients of all ages, most reports have suggested that children and infants are the most at risk for infection by HBoV. In the present study, 406 children with acute respiratory infection in the Gansu Province, China, were examined for the presence of HBoV, with associated clinical presentations and epidemiological characteristics. A phylogenetic analysis of the HBoV NP-1 gene was also undertaken.

Li-shu Zheng, Xin-hui Yuan, Zhi-ping Xie, and Yu Jin contributed equally to this work.

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MATERIALS AND METHODS

Patients and Specimens

Between December 1, 2006, and November 30, 2007, 406 children younger than 14 years of age with acute respiratory infection, who had been admitted to the First Hospital of Lanzhou University, Gansu Province, China, were enrolled in the study after obtaining informed consent from their parents or guardians. The study was approved by the Ethics Committee of the hospital. Nasopharyngeal aspirate specimens were collected from all patients and transported immediately to the laboratory at the National Institute for Viral Disease Control and Prevention, China Center for Disease Control, and stored at −80°C until processing further. Demographic data and clinical findings were recorded on a standard form.

DNA/RNA Extraction and Viral Detection

DNA and RNA were extracted from 0.2 ml of each nasopharyngeal aspirate specimen using QIAamp viral DNA and QIAamp viral RNA mini-kits (Qiagen, Hilden, Germany). Reverse transcription of dsRNA was carried out using SS viral reverse transcriptase and random hexamer primers (Invitrogen, Carlsbad, CA), and PCR amplification was performed with rTaq DNA polymerase (TaKaRa Biotechnology, Dalian, China). Screening of HBoV used routine PCR methods. HBoV primers 188F (5′-GAGCTCTGTAAGTACTATTAC-3′) and 542R (5′-CTCTGTGTTGACTGAATACAG-3′) targeting the NP-1 protein gene and producing a 354-bp amplicon were used, as described and modified by Allander et al. [2005]. Additionally, specimens infected with HBoV were screened for human metapneumovirus (hMPV), respiratory syncytial virus (RSV), influenza virus types A and B, parainfluenza virus types 1–3, human rhinoviruses, and human coronaviruses (HCoV 229E, HCoV OC43, HCoV NL63, and HKU1) using a standard reverse transcription-PCR technique [Bastien et al., 2005; Bellau-Pujol et al., 2005; Vabret et al., 2001, 2006] and for adenovirus using traditional PCR methods [Hierholzer et al., 1993]. Amplification products of different viruses were identified using gel electrophoresis and ethidium bromide staining. To validate the amplification process and exclude carryover contamination, positive and negative controls were included for each PCR.

Real-Time PCR for HBoV

A TaqMan real-time PCR experiment targeting the NP-1 region of HBoV was conducted to quantify viral load. Briefly, 2.5 μl genomic DNA was amplified in a 25-μl PCR mixture containing 12.5 μl ABI TaqMan 2× PCR Master mix, 0.5 μl of each primer (20 pM), 0.125 μl probe (at 10 pM), and 8.875 μl ddH_2O. The primer sequences used were NP1-F: AGAGGCTCGGGCTCATATCA (2478–2497, DQ000469) and NP1-R: CACTTGGTCTGAGGTCTTCGAA (2537–2558, DQ000469), and the probe was TET-AGGAACACCCAATCARCCACCTATCGTCT-TAMRA (2500–2528, DQ000469). The cycling conditions included initial incubation at 50°C for 2 min and 95°C for 15 min, followed by 55 cycles of 95°C for 15 s and 60°C for 1 min. Plasmid pGEM-T/NP-1, containing the target sequence, was constructed and used as a positive control for calculation of the copy number.

Sequencing and Phylogenetic Analysis

All HBoV-positive PCR products were purified using the QIAquick PCR purification kit (Qiagen) and cloned into the pGEM-T Easy vector (Promega, Madison, WI). Subsequently, all NP-1 partial gene sequences of the 29 HBoV strains were sequenced by Invitrogen (Shanghai, China) and deposited in the GenBank database under the accession numbers FJ548896 to FJ548924. Using the DNAStar software package, the 354-bp NP-1 sequences were aligned with other sequences available in GenBank (including HBoV reference strains ST1, ST2, parvovirus B19, bovine parvovirus, canine minute virus, and virus strains isolated from several countries). A neighbor-joining tree was constructed using the MEGA 3.1 program.

Statistical Analysis

The significance of rate and viral load differences among various groups was tested using Fisher's exact test and the Mann–Whitney test. Analyses were performed using SPSS 16.0 software.

RESULTS

Epidemiology of HBoV

A total of 406 nasopharyngeal aspirate samples were obtained from 406 children with acute respiratory tract infection, the male/female ratio was 242:164 (1.48:1) and the median age was 2 years, 5 months (age range = 1 day to 14 years) [Yuan et al., 2008]. HBoV was detected by PCR in 29 (7.1%) of the 406 nasopharyngeal aspirate specimens. Eighteen (62%) of the patients infected with HBoV were male and 11 (38%) were female. The infected patients ranged in age from 24 days to 9 years, and children ≤5 years of age accounted for 89.7% (26/29) of the children infected with HBoV. Children 25–36 months of age had the highest infection rate (12.8%) and those 0–6 months of age had the lowest infection rate (4.9%; Fig. 1). HBoV was detected in every month of the study year except March, July, and November. The highest numbers of positive cases were in December and April (9 positive specimens each), whereas the peak incidence of 20.4% (9/44) was in May (Fig. 2).

HBoV and Coinfection

When tested for other respiratory viruses, 55.2% (16/29) of the patients infected with HBoV were found to be coinfected with other viruses, of which RSV was the most common, accounting for 9 of 16 (56.2%) of coinfections. Furthermore, three patients were

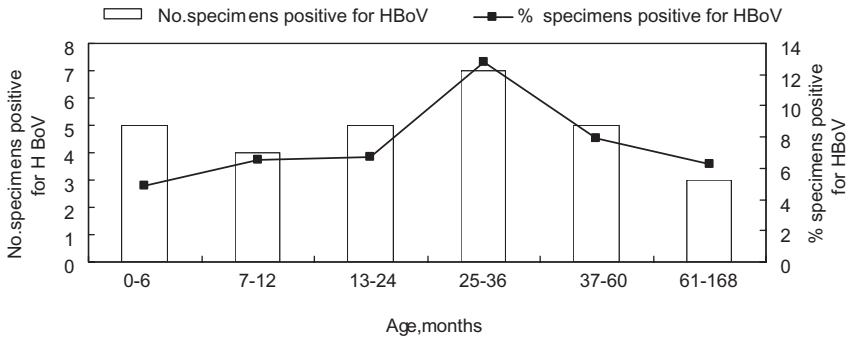

Fig. 1. Age distribution of HBoV in children with acute respiratory tract infection during a 1-year study period.

coinfected with a third virus: hMPV, HCoV NL63, or influenza virus A. Coinfection with human rhinoviruses was observed in four specimens (25.0%), hMPV in one (6.3%), HKU1 in one (6.3%), and parainfluenza virus type 1 in one (6.3%). Monoinfection with HBoV was detected in 13 patients with definite clinical evidence of respiratory infection (Table I).

HBoV Viral Load

The sensitivity of the PCR assay was 100 copies per reaction, as determined by dilutions of the pGEM-T/NP-1 plasmid. Among the HBoV-positive specimens, HBoV genome copies ranged from 5.80×10^2 to 9.72×10^8 copies/ml nasopharyngeal aspirate by real-time PCR. The HBoV genome copy number was 9.73×10^7 copies/ml nasopharyngeal aspirate in children infected with HBoV only, slightly higher than the 8.95×10^6 copies/ml nasopharyngeal aspirate in those with coinfection, but not significantly different ($P = 0.148$; Mann–Whitney test). The HBoV genome copy numbers were 1.30×10^8 and 1.76×10^7 copies/ml nasopharyngeal aspirate in the groups with acute upper respiratory infection and lower respiratory infection, respectively; however, these were not significantly different ($P = 0.354$; Mann–Whitney test; Table II). These results indicated that the viral load of HBoV was not associated with coinfection or the severity of the diseases. The detection limit of the assay for HBoV DNA was 10 genome equivalents per reaction. None of the negative control samples showed false-positive reactions in duplicate.

Clinical Characteristics of HBoV in Children

Acute upper respiratory infection was observed in eight patients (27.6%), acute bronchopneumonia in eight (27.6%), bronchopneumonia in six (20.7%), asthma and bronchopneumonia in three (10.3%), pneumonia in two (6.9%), and acute bronchitis in two patients (6.9%). There were 3 patients with acute upper respiratory infection and 10 patients with lower respiratory infection in the group infected with HBoV only and 5 patients with acute upper respiratory infection and 11 patients with lower respiratory infection in the HBoV coinfection group, but coinfection with HBoV did not appear to affect the severity of the disease ($P = 0.291$; Table II). The most common symptom was cough, which occurred in 27 patients (93.1%). Other clinical presentations included fever (n = 17, 58.6%), crepitations (n = 15, 51.7%), rhinitis (n = 11, 37.9%), wheezing (n = 6, 20.7%), vomiting (n = 2, 6.9%), and dyspnea (n = 2, 6.9%; Table I). Differences in the frequency of cough and crepitations between the HBoV monoinfection and coinfection groups were not statistically significant ($P = 0.512$ and 0.253, respectively), but the frequency of fever was significantly higher in the group infected with HBoV only than in the coinfection group ($P = 0.012$; Table II).

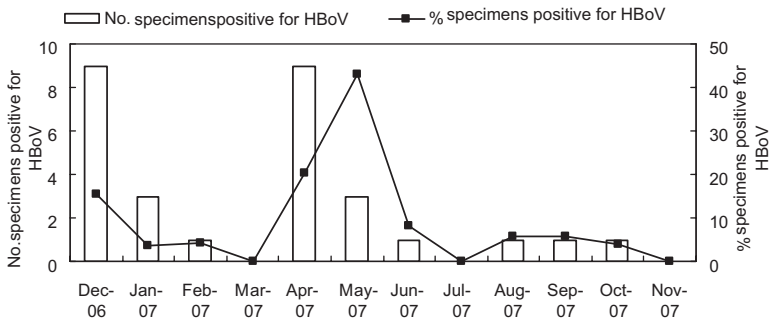

Fig. 2. Seasonal distribution of HBoV in children with acute respiratory tract infection during a 1-year study period.

TABLE I. Clinical Presentations and Demographic Information of the 29 HBoV-Positive Patients

Patient	Age	Sex	Coinfection	Diagnosis	Signs/symptoms	HBoV copy/ml NPA
Lz1	6yr	M	None	Bronchopneumonia	Rhinitis, fever, cough	2.57E + 03
Lz2	2yr, 5m	F	None	Acute upper respiratory tract infection	Fever, cough	1.76E + 04
Lz3	1m	M	RSV + hMPV	Acute bronchopneumonia	Cough, crepitations in the lungs	3.46E + 04
Lz4	3yr, 5m	M	None	Acute bronchopneumonia	Fever, cough, crepitations in the lungs	3.74E + 04
Lz13	6yr	M	hRV	Bronchopneumonia	Cough, crepitations in the lungs	6.76E + 03
Lz25	1yr	M	None	Acute upper respiratory tract infection	Rhinitis, fever, cough	6.60E + 07
Lz28	2yr	M	None	Acute bronchopneumonia	Rhinitis, fever, cough, vomit	1.74E + 05
Lz37	3yr, 6m	F	hMPV	Acute upper respiratory tract infection	Rhinitis, fever, cough	1.91E + 04
Lz110	5yr	M	None	Pneumonia	Cough, wheezing, crepitations in the lungs	5.57E + 03
Lz112	2yr, 9m	F	HKU1	Acute upper respiratory tract infection	Rhinitis, cough	1.39E + 04
Lz118	1yr	M	hRV	Acute upper respiratory tract infection	Rhinitis, cough	1.24E + 04
Lz123	1m	M	hRV	Bronchopneumonia	Rhinitis, cough, crepitations in the lungs	7.66E + 02
Lz160	1yr, 7m	M	RSV	Acute upper respiratory tract infection	Rhinitis, cough	7.68E + 03
Lz162	2yr, 2m	M	None	Bronchopneumonia	Fever, cough, wheezing, crepitations in the lungs, diarrhea	1.99E + 05
Lz166	3m	F	RSV	Acute upper respiratory tract infection	Fever, cough	5.67E + 03
Lz169	1yr	M	RSV	Asthmatic bronchopneumonia	Fever, cough, crepitations in the lungs	1.07E + 04
Lz171	3yr	F	RSV	Acute bronchopneumonia	Fever, cough, wheezing	5.80E + 02
Lz173	2yr	M	None	Acute bronchopneumonia	Fever, cough, crepitations in the lungs	9.33E + 03
Lz174	4yr	F	RSV	Acute bronchopneumonia	Rhinitis, fever, cough, dyspnea,	1.31E + 04
Lz175	4yr	M	RSV	Asthmatic bronchopneumonia	Rhinitis, cough, crepitations in the lungs	6.38E + 03
Lz179	3yr	M	RSV + NL63	Acute bronchopneumonia	Rhinitis, cough, crepitations in the lungs	4.90E + 03
Lz184	24 days	M	hRV	Pneumonia	Dyspnea, crepitations in the lungs	4.86E + 04
Lz190	1yr, 3m	F	None	Acute Bronchitis	Fever, cough	2.88E + 04
Lz196	7m	F	RSV + IFVA	Acute Bronchitis	Fever, cough, crepitations in the lungs	1.55E + 04
Lz197	2yr	F	None	Asthmatic bronchopneumonia	Fever, cough, wheezing, crepitations in the lungs	7.71E + 03
Lz263	3yr	F	None	Acute upper respiratory tract infection	Fever, cough	9.72E + 08
Lz285	6m	F	PIV1	Bronchopneumonia	Cough, wheezing, crepitations in the lungs	1.43E + 08
Lz302	9yr	M	None	Bronchopneumonia	Cyanosis, shortness of breath, nausea, vomit, respiratory failure	3.96E + 03
Lz327	3yr	M	None	Acute bronchopneumonia	Fever, cough, wheezing, crepitations in the lungs	2.26E + 08

M, male; F, female; RSV, respiratory syncytial virus; hMPV, human metapneumovirus; hRV, human rhinovirus; HKU1, human coronavirus HKU1; NL63, human coronavirus NL63; IFVA, influenza virus A; PIV1, parainfluenza virus 1; yr, years; m, months.

NP-1 Gene Polymorphism of HBoV

Partial NP-1 coding sequences (354 bp) by PCR were aligned with the HBoV prototype strains: ST1 and ST2 (GenBank accession numbers DQ000495, DQ000496). Alignment of the sequences obtained in the present study with the HBoV prototype strains showed only minor differences, with a nucleotide identity of 98.3–99.7% and an amino acid identity of 95.7–99.1%. The nucleotide identity and amino acid identity were 98–100% and 95.5–100% among the 29 HBoV-positive strains isolated. Phylogenetic

TABLE II. Statistical Analysis of the Clinical Presentations of the 29 HBoV-Positive Patient

Clinical diagnosis and presentation	HBoV monoinfection, no. positive/no. total, n (%)	HBoV coinfection, no. positive/no. total, n (%)	*P*-value[a]
AURI	3/13, 23.1	5/16, 31.3	0.291
Cough	12/13, 92.3	15/16, 93.8	0.512
Fever	11/13, 84.6	6/16, 37.5	0.012
Cracker	6/13, 46.2	9/16, 56.3	0.253

AURI, acute upper respiratory tract infection.
[a]Fisher's exact test.

analysis of these sequences and those from bovine parvovirus, canine minute virus, and B19 indicated that HBoV was related more closely to bovine parvovirus (Fig. 3).

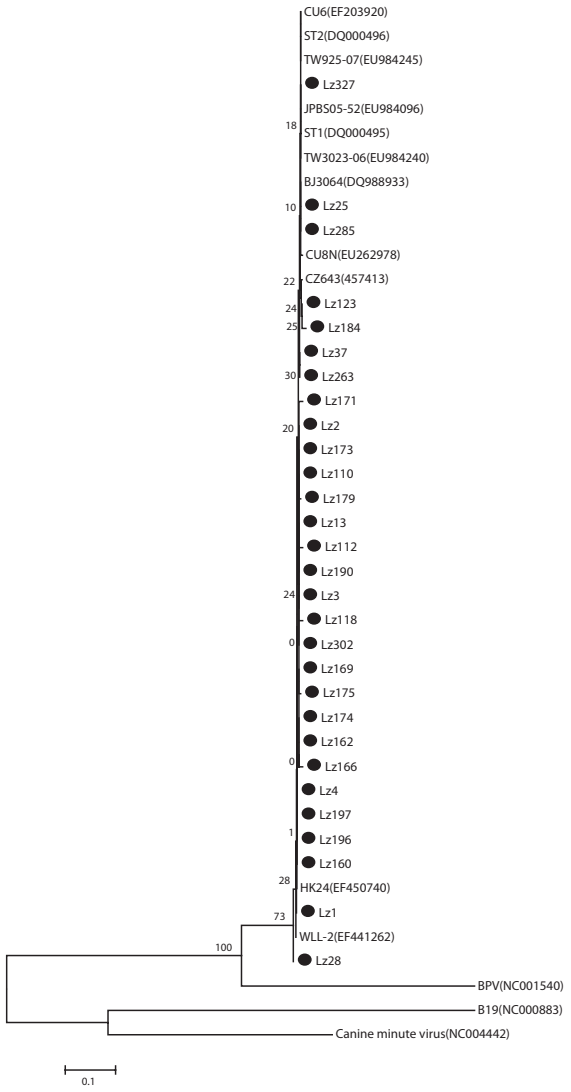

Fig. 3. Phylogenetic analysis of the complete NP-1 nucleotide sequences of HBoV. Phylogenetic trees were constructed by neighbor-joining method by using MEGA 3.1, and bootstrap values were determined by 1,000 replicates. Viral sequences marked with solid circle were generated from the present study, and other reference sequences were obtained from GenBank.

DISCUSSION

Since the discovery of HBoV in 2005, it has been detected frequently worldwide in nasopharyngeal samples [Allander et al., 2005; Arnold et al., 2006; Ma et al., 2006; Weissbrich et al., 2006; Fry et al., 2007; Lau et al., 2007; Pozo et al., 2007; Qu et al., 2007; Fabbiani et al., 2009; Tan et al., 2009; Tozer et al., 2009], serum [Allander et al., 2007], fecal [Lau et al., 2007; Lee et al., 2007; Pozo et al., 2007; Tozer et al., 2009], and urine samples [Pozo et al., 2007] obtained from young children and adults. The epidemiology, clinical characteristics, and polymorphism of HBoV in a non-preselected group of children with acute respiratory infection in the Lanzhou Province, was described.

The incidence of HBoV infection in respiratory illness has been estimated as between 1.5% and 18.9% [Bastien et al., 2006; Allander et al., 2007], mostly in children under the age of 2 years [Allander et al., 2007; Bastien et al., 2007; Catalano-Pons et al., 2007; Kleines et al., 2007; Tan et al., 2009]. Our data indicate that HBoV was present in 7.1% of the children younger than 14 years of age with acute respiratory tract infection and that 89.7% (26/29) of children infected with HBoV were younger than 5 years of age. Thus, the infection rate observed in the present study is consistent with previous reports, although the age group with the highest infection rate (12.8%) was 25–36 months of age. Because of the varying sensitivity of the different methods used for detecting HBoV, the exact contribution of HBoV to acute respiratory infection remains to be evaluated further. Unlike previous reports that HBoV infection was detected predominantly during the winter months [Allander et al., 2005; Kesebir et al., 2006; Weissbrich et al., 2006; Lau et al., 2007; Manning et al., 2007; Pozo et al., 2007] or that the prevalence of infection increased in the spring and summer [Arnold et al., 2006; Choi et al., 2006], it was found that HBoV was detected throughout the year without any clear seasonal fluctuations. The detection of HBoV in different specimens derived from patients avoided unnecessary treatment with antibiotics.

Many reports have indicated that HBoV has high rates of coinfection with other viral and bacterial respiratory pathogens, such as human rhinoviruses, hMPV, adenovirus, RSV, and *Streptococcus* spp. In this study, 55.2% of the patients infected with HBoV were coinfected with other respiratory viruses, and this number would likely have been higher if other

respiratory viruses had been sought. The situation of coinfection was complex, and coinfection rates varied considerably from those in previous research. Coinfection was found in up to 69.2% of patients with HBoV DNA in Israel [Hindiyeh et al., 2008]. Coinfection leading to more severe disease has been described for hMPV and RSV [Greensill et al., 2003]. In the present study, the most prevalent copathogen was RSV in 9 of 16 patients. Patients were divided into acute upper respiratory infection and lower respiratory infection groups, and statistical comparisons indicated that coinfection with HBoV did not affect the severity of the disease. Because of the high coinfection rates, the exact role played by HBoV in respiratory tract disease needs to be considered more precisely.

Different DNA loads of HBoV have been reported in various studies [Allander et al., 2007; Kleines et al., 2007]. Recently, it was reported that the viral load of HBoV in samples from children with HBoV monoinfection was significantly higher than in samples from children with coinfection [Brieu et al., 2008]. However, no statistical difference was found in the genome copies of HBoV between children with HBoV monoinfection versus coinfection; in the current study no correlation was found between the severity of HBoV infection and the viral load in acute upper respiratory infection or lower respiratory infection. The exact relationship between HBoV coinfection, disease severity, and HBoV viral load merits further investigation.

Among the children infected with HBoV, the most common symptom was cough (93.1%); other clinical signs included fever, crepitations in the lung, rhinitis, wheezing, and, less commonly, vomiting, dyspnea, and diarrhea. Unlike cough and crepitations, the frequency of fever was statistically higher in the HBoV monoinfection group than in the coinfection group; the reason remains unclear. Acute upper respiratory infection and acute bronchopneumonia were the most frequent diagnoses. Although HBoV may be a causative agent of respiratory tract infection in children, the relationship between HBoV and acute respiratory infection needs to be investigated further. Additionally, as newly discovered bocaviruses, HBoV2 and HBoV3 were detected in stool samples from children with acute gastroenteritis [Arthur et al., 2009; Kapoor et al., 2009]. The association of HBoV2 and HBoV3 with gastroenteritis, as well as the role of HBoV, HBoV2, and HBoV3 in human disease requires further investigation.

According to the sequence analysis, all of the HBoV strains found in the present study were in the same cluster as the HBoV prototype strains ST1 and ST2, with a DNA sequence homology of 98.3–99.7% and an amino acid identity of 95.7–99.1%. However, nucleotide and amino acid identities were 98–100% and 95.6–100%, respectively, in the 29 HBoV-positive strains isolated. In agreement with previous findings from other countries [Allander et al., 2005; Arnold et al., 2006; Ma et al., 2006; Sloots et al., 2006], the results of the current study show that NP1 represents a conserved region of HBoV and may be suitable for detection of HBoV.

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