Detection and genome analysis of human bocavirus 1-4 from hospitalized children with acute lower respiratory tract infection and symptoms of wheezing in Shanghai

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Abstract. The aim of this study was to elucidate the clinical and molecular epidemiology of human bocavirus (HBoV)1-4 in hospitalized children in Shanghai suffering from acute lower respiratory tract infection with symptoms of wheezing. HBoV1-4 was detected by nested PCR from 275 nasopharyngeal secretion samples collected from hospitalized children. The HBoV-positive DNA sequences were aligned and phylogenetic trees were constructed. The detection rate of HBoV1 was 5.45% (15/275), which was second only to respiratory syncytial virus (RSV). HBoV1 was co-detected with other potential pathogens in most of the samples. No sample was HBoV2-4-positive. Homology analysis of the partial *VP1/VP2*, *NP1* and *NS1* sequences revealed that these genes belonged to the same HBoV1 genotype. Furthermore, the phylogenetic analysis indicated that these epidemic strains clustered on one independent branch. Our results demonstrate that HBoV1 may be one of the common pathogens responsible for the hospitalization of children with acute lower respiratory tract infection and symptoms of wheezing in Shanghai. HBoV1 infection cases are often associated with other pathogens. The viral strains responsible for winter epidemics circulating among children in Shanghai belonged to the same genotype of HBoV1; thus, they may be derived from one common ancestor.

Introduction

Human bocavirus (HBoV) has been detected in both adult and children respiratory tract samples since its discovery in 2005 (1-3). The HBoV genome contains 3 open reading frames (ORFs), encoding 2 non-structural proteins (NS1 and NP1) and 2 viral capsid proteins (VP1 and VP2; *VP2* overlaps with *VP1*, but they have different start codes). HBoV strains with larger than 10% *VP1* nucleotide differences (VPD) were distinguished as different genotypes, while VPD between 5% and 10% was typing criteria for different subgenotypes. According to the typing criteria, the HBoV discovered in 2005 was termed HBoV1 (4,5). Subsequently, 3 more genotypes of HBoV (HBoV2-4) were found from human excrement (4-6). HBoV2 can be divided into 2 subgenotypes, HBoV2A and HBoV2B. It has been shown that inter- and intra-genotype recombinations are present among the bocavirus (7).

In humans, the level of anti-HBoV1 antibody increases 2 months after birth and then undergoes a continuous decline before the age of 6 months. A continuous increase in anti-HBoV1 antibody levels can be observed from the age of 6 months to 6 years. By the age of 2, approximately 80% of children have been infected by HBoV1-4 (8-10). Serological detection has revealed that HBoV has a long-term worldwide prevalence among individuals of all ages. Recent studies have indicated that HBoV1 can be cultivated in human respiratory epithelial cells *in vitro* (11,12). However, no animal infection model has been established thus far; HBoV could not be confirmed as a human pathogen according to the Koch theory (13-15). Some reports have pointed out that HBoV1 is closely related to symptoms of wheezing in children (8,16), while other studies have considered HBoV1 as a passerby or co-infector virus (17,18). Evidence has shown that HBoV1-4 may also be involved in human gastrointestinal tract infections (1,19-21). In our previous study, HBoV1 was found to be closely associated with acute respiratory tract infection in children in Shanghai and most of the infected children had symptoms of wheezing (22). Whether HBoV2-4 is involved in acute respiratory tract infection with symptoms of wheezing is unknown and the genetic evolutionary relation of the epidemic HBoV1-4 strains has not been determined yet.

During the high-occurrence season for acute respiratory tract infections (wintertime, from December 2012 to February 2013), we collected samples of nasopharyngeal secretion (NPS) from hospitalized children with acute lower respiratory tract infection and symptoms of wheezing. We detected the existence of HBoV1-4 in the samples by nested PCR. The DNA sequences of *VP1/VP2*, *NP1* and *NS1* in the HBoV-positive samples were further amplified, sequenced and aligned for phylogenetic tree construction. Our results revealed that the HBoV strains detected from the positive samples belonged to one genotype of HBoV1 and it was suggested that all the strains were derived from one common ancestor.

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Table I. The general nested PCR primer pairs for HBoV1-4 *VP1/VP2* and HBoV1 *NP1* and *NS1*.

Gene name	Primer name	Primer sequence
HBoV1-4 *VP1*	AK-VP-F1	5'-CGCCGTGGCTCCTGCTCT-3'
	AK-VP-R1	5'-TGTTCGCCATCACAAAAGATGTG-3'
HBoV1-4 *VP2*	AK-VP-F2	5'-GGCTCCTGCTCTAGGAAATAAAGAG-3'
	AK-VP-R2	5'-CCTGCTGTTAGGTCGTTGTTGTATGT-3'
HBoV1 *NP1*	HBoV_2204F	5'-GAGACATCGCAAGTGGACTAT-3'
	HBoV_3101R	5'-TTGAGCAGCGCGATCAGCGTTA-3'
	HBoV_2321F	5'-GCACAGCCACGTGACGAAGATGA-3'
	HBoV_3056R	5'-GGATTAAATGGCCCAAGATA-3'
HBoV1 *NS1*	Adel-OF	5'-AGGTAAAACAAATATTGCAAAGGCCATAGTC-3'
	Adel-OR	5'-TGGGAGTTCTCTCCGTCCGTATC-3'
	Adel-IF	5'-AGGGTTTGTCTTTAACGATTGCAGACAAC-3'
	Adel-IR	5'-TATACACAGAGTCGTCAGCACTATGAG-3'

Materials and methods

This study was approved by the Ethics Committee of the Children's Hospital of Fudan University, Shanghai, China. The 275 NPS samples were collected upon the agreement of the guardians of the infected children.

Materials. A total of 275 NPS samples were collected from children with symptoms of wheezing (within 48 h of hospitalization during the period between December 2012 and February 2013) with acute lower respiratory tract infection. The patients (158 males and 117 females) were aged between 1 month to 8 years; there were 56 cases of bronchitis and 219 cases of pneumonia (including 5 cases of severe pneumonia). All patients had symptoms of coughing and wheezing and a chest radiograph indicated the presence of bronchitis and pneumonia. Of the 275 patients, 136 had a fever (>38˚C). Patients displaying symptoms of coughing and wheezing after 48 h of hospitalization or with a past history of asthma were excluded from this study.

HBoV1-4 detection. Viral RNA was extracted according to the instructions provided with the Viral Genomic DNA/RNA Extraction kit [Cat. no. DP 315; Tiangen Biotech (Beijing) Co., Ltd., Beijing, China]. The general nested PCR primer pairs were designed for HBoV1-4 *VP1/VP2* and HBoV1 *NP1* (1,4,23) (Table I). The available PCR reaction conditions and programs were used in our study (1,4,6,23-25). The HBoV plasmid was kindly provided by Professor Zhou Rong from Guangzhou Medical University, Guangzhou, China.

Alignment analysis of HBoV1 VP1/VP2, NP1 and NS1 DNA sequences. The DNA sequences obtained were sequenced and aligned using online BLAST software. The DNA sequences were analyzed and phylogenetic trees were generated using MEGA5.1.

Detection of other common respiratory tract viruses and pathogens. The direct immune fluorescence technique (Respiratory Panel IFA Kit; Chemion) was used for antigen detection for respiratory syncytial virus (RSV), adenovirus (ADV), influenza virus (IFV)-A, IFV-B and parainfluenza virus (PIV)1-3. The fluorescent PCR and bacteria cultivation results of *Mycoplasma pneumoniae* and *Chlamydia trachomatis* for the 275 NPS samples were also obtained.

Results

Detection of HBoV1-4. The nested PCR results revealed that 15 of the 275 NPS samples were HBoV1-positive; the detection rate was 5.45%. With the use of common primers for HBoV1-4 *VP1/VP2*, HBoV1 *NP1* and HBoV1 *NS1,* 15, 15, and 8 positive samples were detected, respectively, by nested PCR. Seven samples with positive signals of *VP1/VP2* and *NP1* failed to yield nested RT-PCR products using *NS1* primers. The clinical information of the 15 HBoV1-positive samples is summarized in Table II.

All the PCR products obtained were sequenced. All the sequences were pooled together with the HBoV1 whole genome (available in GeneBank) for homology analysis using ClustalW (MEGA5.1). The detailed homology information for *VP1/VP2*, *NP1* and *NS1* is shown in Figs. 1-3, respectively.

Sequence homology analysis of the HBoV1 VP1/VP2 gene. The 15 HBoV1 strains in our study showed a maximum homology difference of 1.7% with previously discovered strains [strains reported in 2010 and 2011 in Guangzhou: GZ2010-1(JN128956), GZ2010-03(JN128953), GZ2011-01(JN128954) and GZ2011-04(JN128955); strains reported in 2012 in Guangzhou: GZ4785(JN794565) and GZ9081(JN794566); strains reported in 2012 in the US: KU3(JQ411251)]. A minimum difference of 28.1% was shown between the 2009 Australian HBoV2 strains [W298(FJ948860) and W208(EU082214)] and the newly discovered strains. A minimum difference of 30.8% was shown between the 2009 Australian HBoV3(NC_012564) and the new HBoV1 strains. A minimum difference of 30.6% was shown between the 2010 American HBoV4(NC_012729) and the new HBoV1 strains. The maximum difference among the 15 new HBoV1 strains was 1.3%, which indicated that they belonged to the same genotype (Fig. 1).

Table II. Clinical information of children infected by HBoV1.

No.	Age	Gender	Clinical diagnosis	Diarrhea	Co-infected pathogens
1	3 Months	Female	Pneumonia	Yes	RSV
2	11 Months	Male	Pneumonia	Yes	RSV, hMPV
3	3 Years	Male	Bronchitis	No	Hi
4	2 Years	Female	Pneumonia	Yes	
5	3 Years	Female	Pneumonia	No	ADV, MC
6	8 Months	Female	Bronchitis	Yes	RSV
7	6 Years	Male	Pneumonia	No	MP
8	2 Years	Male	Bronchitis	No	RSV
9	8 Years	Male	Pneumonia	No	MP, Strep
10	11 Months	Male	Pneumonia	No	RSV, Hi
11	2 Years	Female	Pneumonia	Yes	IAV
12	8 Months	Male	Bronchitis	Yes	
13	3 Years	Female	Pneumonia	No	RSV
14	4 Years	Female	Pneumonia	No	ADV
15	2 Years	Male	Pneumonia	Yes	

RSV, respiratory syncytial virus; hMPV, human metapneumovirus; ADV, adenovirus; IAV, influenza A virus; Hi, *Haemophilus influenzae*; MC, *Moraxella catarrhalis*; Strep, *Streptococcus pneumoniae*; MP, *Mycoplasma pneumoniae*.

GZ2010-1																										
GZ2010-03	0.004																									
GZ2011-01	0.006	0.002																								
GZ2011-04	0.002	0.002	0.004																							
GZ4785	0.004	0.000	0.002	0.002																						
GZ9081	0.017	0.013	0.015	0.015	0.013																					
KU3	0.007	0.004	0.006	0.006	0.004	0.017																				
W208	0.293	0.298	0.298	0.295	0.298	0.298	0.304																			
W298	0.281	0.287	0.287	0.284	0.287	0.281	0.292	0.019																		
HBoV3	0.324	0.324	0.321	0.327	0.324	0.318	0.327	0.139	0.137																	
HBoV4	0.299	0.305	0.305	0.302	0.305	0.301	0.310	0.143	0.138	0.122																
SH1	0.015	0.011	0.013	0.013	0.011	0.025	0.015	0.301	0.290	0.317	0.315															
SH2	0.011	0.007	0.009	0.009	0.007	0.021	0.011	0.295	0.284	0.311	0.309	0.004														
SH3	0.011	0.007	0.009	0.009	0.007	0.021	0.011	0.295	0.284	0.311	0.309	0.004	0.000													
SH4	0.011	0.011	0.013	0.009	0.011	0.025	0.015	0.296	0.285	0.317	0.309	0.004	0.004	0.004												
SH5	0.013	0.013	0.015	0.011	0.013	0.027	0.017	0.299	0.288	0.320	0.313	0.004	0.006	0.006	0.002											
SH6	0.011	0.011	0.013	0.009	0.011	0.025	0.015	0.296	0.285	0.317	0.309	0.004	0.004	0.004	0.000	0.002										
SH7	0.015	0.011	0.013	0.013	0.011	0.025	0.015	0.305	0.293	0.321	0.315	0.006	0.006	0.006	0.006	0.007	0.006									
SH8	0.015	0.011	0.013	0.013	0.011	0.025	0.015	0.301	0.290	0.317	0.315	0.007	0.004	0.004	0.007	0.009	0.007	0.009								
SH9	0.011	0.007	0.009	0.009	0.007	0.021	0.011	0.295	0.284	0.311	0.309	0.004	0.000	0.000	0.004	0.006	0.004	0.006	0.004							
SH10	0.015	0.011	0.013	0.013	0.011	0.025	0.015	0.305	0.293	0.321	0.315	0.006	0.006	0.006	0.006	0.007	0.006	0.000	0.009	0.006						
SH11	0.015	0.011	0.013	0.013	0.011	0.021	0.015	0.293	0.281	0.308	0.306	0.007	0.004	0.004	0.007	0.009	0.007	0.009	0.007	0.004	0.009					
SH12	0.017	0.013	0.015	0.015	0.013	0.023	0.013	0.301	0.284	0.316	0.314	0.009	0.006	0.006	0.009	0.011	0.009	0.011	0.009	0.006	0.011	0.009				
SH13	0.019	0.015	0.017	0.017	0.015	0.025	0.015	0.304	0.287	0.320	0.318	0.011	0.007	0.007	0.011	0.013	0.011	0.013	0.011	0.007	0.013	0.011	0.002			
SH14	0.017	0.013	0.015	0.015	0.013	0.023	0.013	0.301	0.284	0.316	0.314	0.009	0.006	0.006	0.009	0.011	0.009	0.011	0.009	0.006	0.011	0.009	0.000	0.002		
SH15	0.011	0.007	0.009	0.009	0.007	0.021	0.011	0.295	0.284	0.311	0.309	0.004	0.000	0.000	0.004	0.006	0.004	0.006	0.004	0.000	0.006	0.004	0.006	0.007	0.006	

Figure 1. Sequence homology of *VP1/VP2* for the 15 HBoV1 strains and whole genome sequence of HBoV1 (available in GenBank). ClustalW (MEGA5.1) was used for sequence homology comparison and calculation.

Sequence homology analysis of the HBoV1 NP1 gene. The homology differences among the newly discovered HBoV strains and previously discovered strains were also calculated based on the *NP1* sequences. A maximum difference in homology of 2.3% was observed between the 15 HBoV1 strains and the previously discovered strains (GZ2010-1, GZ2010-03, GZ2011-01, GZ2011-04, GZ4785, GZ9081 and KU3). The minimum difference between W298 and W208 and the newly discovered HBoV1 strains was 27.8%; the minimum difference between the new strains and HBoV3 was 15.2%; the minimum difference between the new strains and HBoV4 was 27.3%; the maximum difference between the 15 new HBoV1 strains was 1. 8%, which indicated that they belonged to the same genotype (Fig. 2).

Sequence homology analysis of the HBoV1 NS1 gene. The homology differences among the newly discovered HBoV strains and the previously discovered strains were also calculated based on the 8 *NS1* sequences. A maximum difference in homology of 3.6% was observed between the 8 HBoV1 strains and the previously discovered strains (GZ2010-1, GZ2010-03, GZ2011-01, GZ2011-04, GZ4785, GZ9081 and KU3). A minimum difference of 19.5% was shown among the new strains and W298

GZ2010-1																								
GZ2010-03	0.006																							
GZ2011-01	0.003	0.003																						
GZ2011-04	0.001	0.004	0.001																					
GZ4785	0.001	0.004	0.001	0.003																				
GZ9081	0.006	0.008	0.006	0.007	0.004																			
KU3	0.001	0.004	0.001	0.003	0.000	0.004																		
W208	0.272	0.272	0.272	0.274	0.270	0.268	0.270																	
W298	0.274	0.274	0.274	0.276	0.272	0.270	0.272	0.011																
HBoV3	0.153	0.155	0.153	0.155	0.152	0.153	0.152	0.263	0.257															
HBoV4	0.268	0.268	0.268	0.270	0.266	0.264	0.266	0.112	0.108	0.271														
SH1	0.008	0.008	0.006	0.007	0.007	0.011	0.007	0.278	0.280	0.159	0.274													
SH2	0.014	0.014	0.011	0.013	0.013	0.017	0.013	0.282	0.284	0.157	0.278	0.010												
SH3	0.015	0.018	0.015	0.017	0.014	0.015	0.014	0.281	0.283	0.159	0.280	0.013	0.010											
SH4	0.013	0.015	0.013	0.014	0.011	0.015	0.011	0.285	0.286	0.159	0.280	0.010	0.007	0.006										
SH5	0.014	0.017	0.014	0.015	0.013	0.017	0.013	0.287	0.289	0.162	0.283	0.011	0.010	0.013	0.007									
SH6	0.011	0.014	0.011	0.013	0.010	0.014	0.010	0.283	0.285	0.157	0.278	0.008	0.007	0.006	0.003	0.007								
SH7	0.011	0.014	0.011	0.013	0.010	0.014	0.010	0.283	0.285	0.160	0.279	0.007	0.008	0.007	0.004	0.008	0.004							
SH8	0.015	0.018	0.015	0.017	0.014	0.015	0.014	0.277	0.279	0.152	0.273	0.013	0.011	0.010	0.010	0.015	0.008	0.011						
SH9	0.011	0.011	0.011	0.013	0.010	0.014	0.010	0.278	0.280	0.155	0.274	0.010	0.008	0.010	0.007	0.008	0.006	0.008	0.013					
SH10	0.017	0.020	0.017	0.018	0.015	0.017	0.015	0.283	0.285	0.160	0.283	0.014	0.013	0.006	0.008	0.011	0.006	0.010	0.014	0.010				
SH11	0.017	0.017	0.014	0.015	0.015	0.020	0.015	0.291	0.293	0.164	0.287	0.011	0.007	0.008	0.008	0.010	0.006	0.010	0.011	0.010	0.008			
SH12	0.020	0.023	0.020	0.021	0.018	0.020	0.018	0.289	0.291	0.166	0.284	0.018	0.015	0.010	0.013	0.014	0.011	0.014	0.014	0.013	0.010	0.011		
SH13	0.013	0.015	0.013	0.014	0.011	0.013	0.011	0.280	0.282	0.159	0.276	0.010	0.010	0.006	0.006	0.010	0.004	0.007	0.011	0.004	0.006	0.008	0.008	
SH14	0.015	0.018	0.015	0.017	0.014	0.015	0.014	0.285	0.287	0.166	0.281	0.011	0.014	0.007	0.010	0.010	0.007	0.006	0.013	0.011	0.007	0.007	0.011	0.007
SH15	0.015	0.018	0.015	0.017	0.014	0.018	0.014	0.283	0.285	0.157	0.279	0.014	0.011	0.013	0.010	0.013	0.007	0.008	0.013	0.011	0.010	0.010	0.014	0.010	0.008

Figure 2. Sequence homology of *NP1* for the 15 HBoV1 strains and whole genome sequence of HBoV1 (available in GenBank). ClustalW (MEGA5.1) was used for sequence homology comparison and calculation.

GZ2010-1																								
GZ2010-03	0.010																							
GZ2011-01	0.008	0.014																						
GZ2011-04	0.008	0.014	0.000																					
GZ4785	0.008	0.014	0.000	0.000																				
GZ9081	0.008	0.014	0.000	0.000	0.000																			
KU3	0.008	0.014	0.000	0.000	0.000	0.000																		
W208	0.208	0.211	0.205	0.205	0.205	0.205	0.205																	
W298	0.211	0.213	0.207	0.207	0.207	0.207	0.207	0.006																
HBoV3	0.102	0.108	0.092	0.092	0.092	0.092	0.092	0.182	0.185															
HBoV4	0.192	0.200	0.189	0.189	0.189	0.189	0.189	0.083	0.090	0.182														
SH1	0.027	0.033	0.019	0.019	0.019	0.019	0.019	0.195	0.198	0.097	0.192													
SH3	0.028	0.034	0.020	0.020	0.020	0.020	0.020	0.196	0.198	0.097	0.192	0.002												
SH4	0.027	0.033	0.019	0.019	0.019	0.019	0.019	0.195	0.198	0.097	0.187	0.006	0.006											
SH6	0.029	0.035	0.021	0.021	0.021	0.021	0.021	0.198	0.200	0.099	0.189	0.006	0.004	0.002										
SH7	0.030	0.036	0.021	0.021	0.021	0.021	0.021	0.198	0.201	0.099	0.190	0.004	0.002	0.004	0.002									
SH8	0.031	0.038	0.023	0.023	0.023	0.023	0.023	0.200	0.203	0.101	0.192	0.008	0.006	0.004	0.002	0.004								
SH14	0.027	0.033	0.019	0.019	0.019	0.019	0.019	0.195	0.198	0.097	0.187	0.006	0.006	0.000	0.002	0.004	0.004							
SH15	0.036	0.042	0.027	0.027	0.027	0.027	0.027	0.213	0.216	0.108	0.200	0.014	0.016	0.020	0.020	0.018	0.021	0.020						

Figure 3. Sequence homology of *NS1* for the 8 HBoV1 strains and whole genome sequence of HBoV1 (available in GenBank). ClustalW (MEGA5.1) was used for sequence homology comparison and calculation.

and W208; the minimum difference between the new strains and HBoV3 was 9.7%; the minimum difference between the new strains and HBoV4 was 18.7%. The maximum difference between the 8 new HBoV1 strains was 2.1%, which indicated that they belonged to the same genotype (Fig. 3). The 8 HBoV1 strains showed a minimum difference of 10% with HBoV3, which supported the assumption that HBoV3 was derived from the recombination of HBoV1 and HBoV2 (4,25,26).

Phylogenetic tree generation for the HBoV1 gene. The positive PCR products of 15 *VP1/VP2* genes, 15 *NP1* genes and 8 *NS1* genes were pooled for phylogenetic tree construction (Fig. 4). All 3 phylogenetic trees showed that the 15 detected viral strains were scattered on the same branch, which indicated that they belonged to the same HBoV1 genotype.

Detection of other common pathogens. The detection rates for 8 common viruses [RSV, ADV, IFA/B, PIV1-3 and human metapneumovirus (hMPV)] were 44.73, 2.18, 1.45, 1.45 and 3.64%, respectively. The total detection rate was 53.4% (147/275); RSV was the most common pathogen (44.73%) and the detection rate of HBoV1 (5.45%) was second to RSV. The 15 HBoV1-positive samples were co-detected with 6 cases of RSV and 2 cases of hMPV. Thirteen cases of *Mycoplasma pneumoniae* and *Chlamydia trachomatis* were identified by qRT-PCR (4.73%); the positive rate of the bacterial culture was 24.36% (67/275); the co-infection rate was 16.0% (44/275); the total detection rate of all the pathogens was 72.0% (198/275).

Discussion

HBoV is a newly discovered Parvoviridae virus. HBoV particles can be detected in respiratory secretions and digestive excrements. A number of scientists have considered that HBoV is closely connected with human respiratory tract infections. However, there is still controversy as to the involvement of HBoV1-4 in children with symptoms of wheezing. The detection of HBoV DNA or particles in the secretions of patients

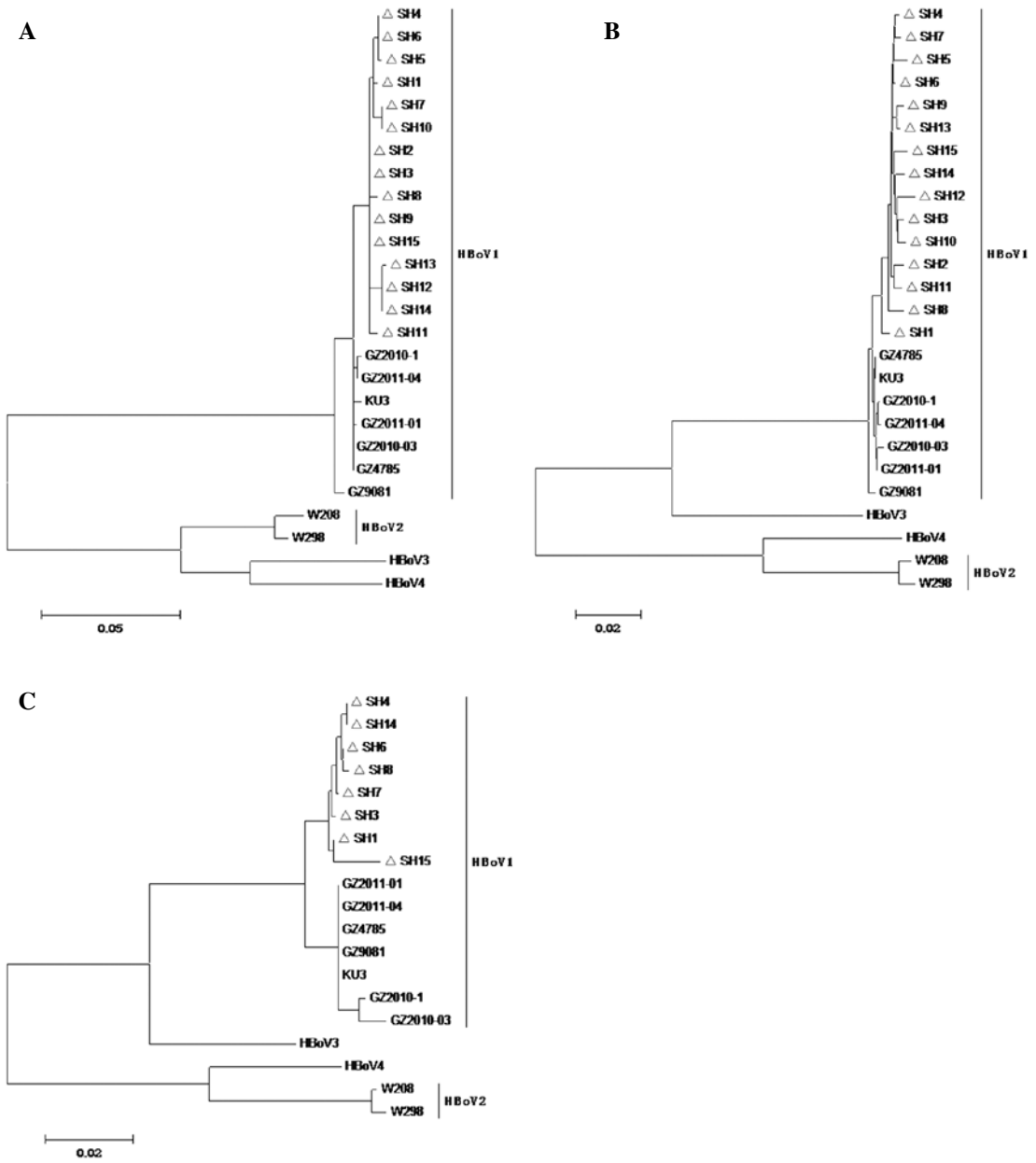

Figure 4. Phylogenetic tree based on nucleotide sequences of *VP1*/*VP2*, *NP1* and *NS1* in the 15 detected HBoV1 strains. (A) Phylogenetic tree for *VP1*/*VP2*; (B) phylogenetic tree for *NP1*; (C) phylogenetic tree for *NS1*. Phylogenetic analysis was conducted using MEGA5.1 (www.megasoftware.com). Genetic distances were calculated with the pairwise distance method. Phylogenetic trees were constructed with the neighbor-joining method.

with symptoms of wheezing and the positive transition of serological HBoV antibody were used in this study as alternative methods of examining the infections, although no animal model was available for an HBoV infection study.

We discovered 15 HBoV1-positive samples from the 275 NPS collected from hospitalized children with acute lower respiratory tract infection with symptoms of wheezing; the detection rate was 5.45%, which was second to RSV. This indicates that HBoV1 may be one of the common pathogens responsible for the hospitalization of children with acute lower respiratory tract infection with symptoms of wheezing in Shanghai. As we did not include a control group with no symptoms, no serological and viremia detection were perrormed in our study; the 15 HBoV1 strains may be co-pathogens only or just viruses being carried by the patients. No HBoV2-4 signals were detected, which indicated that HBoV2-4 could not be the pathogens responsible for the hospitalization of children with acute lower respiratory tract infection with symptoms of wheezing in the winter of 2012; this is consistent with the low detection rate of HBoV2-4 in respiratory tract secretions (27-29). Although 7 HBoV1-positive cases displayed symptoms of diarrhea, it could not be confirmed that the digestive tract removed the viral particles, as no excrement samples of the HBoV1-positive cases were collected and detected. Further studies are required to fully investigate this issue.

The homology differences among the newly discovered HBoV strains and the previously discovered strains were calculated based on the *VP1*/*VP2*, *NP1* and *NS1* sequences. All the results revealed that the 15 new HBoV1 strains belonged to the same genotype (Figs. 1-3). All the 15 strains were scattered on the same branch in the 3 phylogenetic trees (Fig. 4). This indicated that the 15 new HBoV1 strains were very likely derived

from one common HBoV1 ancestor with pathogenicity, which underwent evolution through inter-genotype recombination.

The discovery of HBoV1-4 indicated that HBoV has a feature of genetic diversities. Being consistent with a recent report (29), the analysis of whole genome sequences in GenBank revealed that the gene recombinations occurred not only among HBoV1-4, but also among the members in the same genotypes. The analysis of different ORFs led to the same conclusion that all the newly discovered HBoV1 strains belonged to the same genotype. Further persistent monitoring is required to determine whether the HBoV1 genotype is closely related with digestive tract infections in children with symptoms of wheezing in Shanghai.

In our study, only HBoV1 was detected by nested PCR from the 275 NPS samples collected from the hospitalized children with symptoms of wheezing in the winter of 2012. The detection rate of HBoV1 was 5.45% (15/275), which was second only to RSV. HBoV1 had a high co-infection rate with other potential pathogens in most of the samples. The 15 newly discovered HBoV1 strains belonged to the same subgroup and thus may be derived from one common ancestor. Our results indicated that HBoV1 may be one of the common pathogens responsible for the hospitalization of children with acute lower respiratory tract infection with symptoms of wheezing in Shanghai.

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