

Epidemic and molecular evolution of human bocavirus in hospitalized children with acute respiratory tract infection

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Abstract Human bocavirus (HBoV) is a novel parvovirus, often associated with respiratory tract diseases in children. This study explored the epidemiological characteristics and molecular evolution of HBoV-1 in southeastern China. Nasopharyngeal aspirates were collected from children admitted to hospital with acute respiratory tract infections. HBoV-1 was detected using real-time reverse transcription polymerase chain reaction and further characterized by complete genome sequences analysis. Among the 3,022 recruited children, 386 (12.77 %) were HBoV-1-positive and 300 (77.72 %) had co-detection with other respiratory viruses. Seasonal prevalence peaked in summer. HBoV-1 presence was significantly associated with asthma attack [odds ratio = 1.74; 95 % confidence interval: 1.30, 2.31; $p < 0.001$]. Similar results were obtained when either single detection or co-detection of HBoV-1 was considered, demonstrating the minor impact of co-detection on the clinical characteristics or epidemic pattern.

Phylogenetic analysis based on the complete genome sequences showed that all the HBoV-1 sequences clustered together and no branch was formed that was supported by bootstrap value ≥ 750 . The overall evolutionary rate of the complete genome of HBoV-1 was estimated at 1.08×10^{-4} nucleotide substitutions per site per year (s/s/y) [95 % highest probability density: $(0.40-1.86) \times 10^{-4}$ s/s/y]. Selective pressure analysis showed that all the ω -values were less than 1, suggesting that HBoV-1 was under negative selective pressure. Site-by-site analysis identified the codon site 40 of the VP1 gene under positive selection. In conclusion, our study disclosed the epidemiological and genetic dynamics of HBoV-1 epidemics in southeastern China in the most recent 3 years, the information of which might help to further improve our understanding of HBoV-1 infection and guide better surveillance and control strategies in the future.

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Introduction

Acute respiratory tract infections (ARTI) are a major cause of significant morbidity worldwide, especially in infants and children. Viruses are leading pathogens of ARTI. Although some viruses, such as influenza virus, respiratory syncytial virus (RSV), and human adenovirus (HAdV), are responsible for most ARTI, etiological causes remain unknown in a proportion of ARTI [1].

Human bocavirus (HBoV) is a novel parvovirus first described by Allander et al. in 2005 [2]. It has been classified in the family *Parvoviridae*, subfamily *Parvovirinae*, and genus *Bocavirus* by genetic organization and sequence homology. Other parvoviruses infecting humans included the adeno-associated viruses, the well-known pathogen parvovirus B19, and the recently discovered human parvovirus 4 [3]. Based on the phylogenetic analysis of viral genomes, four species of HBoVs (HBoV1–4) have been identified [4],

among which HBoV-1 was mainly identified in respiratory specimens from children with acute respiratory disease [5–7]. According to previous studies, HBoV-1 has a global distribution, with prevalences varying considerably from 1.5 % to 45.7 % in ARTI patients [5, 6, 8–14]. However, its role as a causative agent of respiratory tract diseases is frequently questioned due to its concurrent detection with other potential pathogens. Possible interactions between HBoV-1 and other viruses remained rarely investigated.

In China, HBoV-1 was detected in children with positive rates ranging from 4.6 % to 31.0 % [6, 14–18]. However, HBoV-1 was co-detected frequently with other viruses in patients with respiratory or enteric infections; therefore, the impact of HBoV-1 on ARTI occurrence remains unclear. The seasonal patterns of HBoV-1 infection in China, especially the surveillance data from the most recent 4 years, are scarcely reported, probably owing to the lack of a systematic and prolonged surveillance.

This study was aimed to explore the epidemiology pattern and clinical characteristics of HBoV-1 infection in Chinese children, as well as the molecular evolutionary pattern, for HBoV-1, by performing a 4-year laboratory surveillance of ARTI cases.

Patients and methods

The laboratory surveillance was performed to detect the viral infection in children with ARTI between June 2009 and May 2013 at the Children's Hospital of Chongqing Medical University, China. The ARTI was determined based on syndromes of cough, rhinorrhea and/or dyspnea, and/or fever of >37.5 °C. Fever caused by known chronic medical conditions was excluded. The pneumonia was defined by the presence of patchy alveolar opacities on chest radiographs, in addition to presenting symptoms of cough, dyspnea (lower chest wall indrawing), or tachypnea (in infants, >50 – 60 breaths/min; in older children, >40 breaths/min). Severe pneumonia was defined as pneumonia plus hypoxemia (maintained $\text{SaO}_2 < 92$ % in air) or rising respiratory and pulse rates with clinical evidence of respiratory distress and exhaustion with or without raised PaCO_2 . The definition of an attack was used as an episode of respiratory symptoms which prompts an urgent consultation with a doctor, is of sufficient severity to prevent the patient working/attending school/performing domestic duties/playing, and results in increased use of anti-asthma medication.

The recruited patients had nasopharyngeal aspirates (NPA) collected the day they were hospitalized. A standardized form was applied to extract the patients' information regarding demographic characteristics, underlying medical conditions, selected laboratory tests, radiographic findings, clinical

manifestation, treatment course, and outcome. Written informed consent was obtained from their parents.

Viral RNA and DNA were extracted from 200 μl of the NPA by using QIAamp® MinElute Virus Spin Kits (Qiagen, Hilden, Germany). The cDNA was synthesized by using the SuperScript®III First-Strand Synthesis System for reverse transcription polymerase chain reaction (RT-PCR) (Invitrogen, Carlsbad, CA, USA). All samples were routinely screened for HBoV-1 with the real-time PCR method [19]. Partial HBoV-1-positive samples were whole-genome sequenced following the method previously described [20].

Genomic sequences were assembled using Lasergene's DNA SeqMan software (version 7.1.0, DNA Star Inc., Madison, WI, USA). Previously published GenBank sequences of HBoV-1 were used for comparison. All alignments and phylogenetic tree construction were performed by the neighbor-joining (NJ) method with 1,000 bootstrap replicates using CLC Genomic Workbench 5.5. Similarities between strains were calculated by using BioEdit version 7.1.3 (North Carolina State University, Raleigh, NC, USA; <http://www.mbio.ncsu.edu/bioedit/bioedit.html>). The substitution rates, the divergence over time, and the time to the most recent common ancestor (TMRCA) of the current HBoVs were estimated using a Bayesian Markov chain Monte Carlo (MCMC) approach, as implemented in the Bayesian Evolutionary Analysis Sampling Trees (BEAST) package version 1.8.0.

The value of ω and the individual site-specific selection pressure were measured by using the single likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), internal fixed effects likelihood (IFEL), and fast unconstrained Bayesian approximation (FUBAR) methods implemented in the hypothesis testing using phylogenies (HyPhy) package performed at Datamonkey online (<http://www.datamonkey.org/>). The sites were confirmed when they were selected by more than two methods. A single breakpoint recombination (SBP) test with both Akaike information criterion (AIC) and Bayesian information criterion (BIC) calculation was conducted. The overall ω -value and 95 % confidence interval (CI) were estimated based on NJ trees. Selective pressure was defined as neutral evolution with $\omega=1$, purifying or negative selection with $\omega < 1$, and positive selection with $\omega > 1$. A p -value of less than 0.10 was considered as the threshold for strong evidence of selection in SLAC, FEL, and IFEL, and posterior probability ≥ 0.9 in FUBAR, respectively.

Statistical analysis was done by the Chi-square test for categorical variables. Logistic regression models were used to explore the factors associated with higher HBoV-1 detection and to determine the association between clinical characteristics and HBoV-1 infection, with clinical disease as the dependent variable and HBoV-1 infection as the independent variable. Odds ratios (ORs) and their 95 % CIs were estimated using maximum likelihood methods. Statistical significance

was set at a *p*-value of less than 0.05. All the tests were performed by using SAS 9.13 (SAS Institute Inc., Cary, NC, USA).

Results

From June 2009 to May 2013, 3,022 NPA samples of hospitalized infants and children were collected. The median age was 8 months (range 1 months to 16 years), and 2,016 (66.71 %) were boys. The median delay before hospital entrance was 9 days (range 1–60 days). The hospitalization duration [mean \pm standard deviation (SD)] was 7 \pm 4 days. Among the 3,022 children, 450 (14.90 %) displayed severe pneumonia and 25 (0.83 %) had severe asthma. In total, 386 (12.77 %) children were positive for HBoV-1. The children aged between 7 and 24 months had higher detection rates of HBoV-1 than children of the other age groups (Table 1). When compared with the >25 months group, the ORs for HBoV-1-positive detection were 1.92 (95 % CI: 1.32, 2.80) for the 7–12 months group and 2.57 (95 % CI: 1.75, 3.76) for the 13–

24 months group after adjustment for the confounding factors. The HBoV-1 detection rate was significantly higher in children having asthma attack (OR = 1.74; 95 % CI: 1.30, 2.31).

Among the 386 children with positive results, 86 (22.28 %) had single HBoV-1 detection and the remaining 300 (77.72 %) were co-detected with other viruses, including human rhinovirus, enterovirus, human adenovirus, influenza virus, metapneumovirus, RSV, parainfluenza virus, and coronaviruses. The most frequently presented co-detection occurred between HBoV-1 with parainfluenza virus (30.05 %), followed by with RSV (28.50 %) and influenza virus (26.17 %). The patients with single detection had a median age of 11 months (range 1 months to 13 years) and 265 (68.65 %) were boys, which were comparable with the subjects with HBoV-1 detection as a whole. The patients with HBoV-1 detection presented clinical manifestations of cough (291, 97.00 %), sputum (226, 75.33 %), and diarrhea (91, 30.33 %). These syndromes were presented with highly comparable frequencies among patients with HBoV-1 single detection [cough (83, 96.51 %), sputum (63, 73.26 %), and diarrhea (26, 30.23 %)]. The occurrence of severe pneumonia

Table 1 Distribution and clinical characteristics of HBoV-1 infections

Factor	HBoV			Negative	Logistic regression model*	
	Positive		<i>p</i> -Value		Positive vs negative	
	Single	Coinfection			OR (95 % CI)	<i>p</i> -Value
Age (months)	86	300	0.286	2,636		
0–6	20 (23.26)	88 (29.1)		1,154 (43.81)	0.87 (0.60, 1.25)	0.443
7–12	23 (26.74)	84 (28.00)		513 (19.48)	1.92 (1.32, 2.80)	<0.001
13–24	30 (34.88)	70 (23.33)		361 (13.71)	2.57 (1.75, 3.76)	<0.001
25–36	5 (5.81)	22 (7.33)		199 (7.56)	1.26 (0.76, 2.10)	0.368
>36	8 (9.30)	36 (12.00)		407 (15.45)	1.00	
Sex (male)	51 (59.30)	214 (71.33)	0.034	1,751 (66.43)	1.10 (0.88, 1.39)	0.406
Clinical manifestation						
Cough	83 (96.51)	291 (97.00)	0.818	2,495 (94.65)	1.87 (1.02, 3.43)	0.044
Sputum	63 (73.26)	226 (75.33)	0.695	1,997 (75.76)	0.98 (0.76, 1.26)	0.871
Diarrhea	26 (30.23)	91 (30.33)	0.986	794 (30.12)	1.05 (0.82, 1.33)	0.726
Clinical diseases						
URTI	17 (19.77)	81 (27.00)	0.174	612 (23.22)	1.13 (0.88, 1.46)	0.335
Bronchitis	8 (9.30)	13 (4.33)	0.073	150 (5.69)	0.94 (0.58, 1.51)	0.788
Pneumonia	73 (84.88)	269 (89.67)	0.219	2,318 (87.94)	1.05 (0.74, 1.48)	0.791
Asthma attack	14 (16.28)	61 (20.33)	0.402	342 (12.97)	1.74 (1.30, 2.31)	<0.001
Wheeze	34 (39.53)	146 (48.67)	0.135	1,159 (43.97)	1.18 (0.94, 1.47)	0.162
Adverse outcome						
Severe pneumonia	12 (16.44)	41 (15.24)	0.946	397 (17.13)	0.95 (0.69, 1.31)	0.742
Severe asthma	2 (14.29)	2 (3.28)	0.216	21 (6.14)	1.79 (0.54, 5.95)	0.342

*The ORs were calculated by adjusting the variables of age, sex, and the infections of influenza virus, HAdV, RSV, PIV, MPV, COV, HRV, and HEV for the clinical manifestation, clinical diseases, and adverse outcome. Logistic regression analysis was applied with clinical disease as the dependent variable and HBoV-1 infection as the independent variable. Odds ratios (ORs) and their 95 % confidence intervals (CIs) were estimated using maximum likelihood method

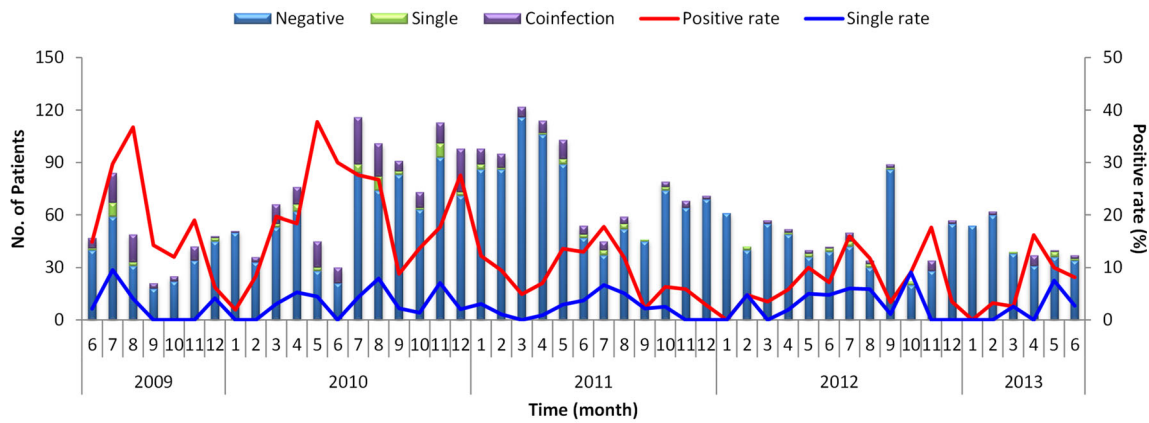


Fig. 1 Distribution of monthly positive rates of HBov-1 infection during 2009–2012

and severe asthma were identified in 53 (13.73 %) and 4 (1.04 %) HBov-1-detected patients, among whom 12 and four had single detection, respectively, which is comparable with the patients with co-detection.

The monthly positive rates of HBov-1 displayed specific seasonal distribution, as plotted in Fig. 1. One seasonal peak was observed in summer, demonstrated by remarkably higher infection rates during May–August. An exception was noticed in 2010, when another low peak was also observed in the winter season from November to December. When the HBov-

1 single infection was plotted, a similar seasonal pattern was derived, with a clearly increased activity of HBov demonstrated in summer.

The phylogenetic tree based on 5,157 bp of the whole genome sequences showed that all the HBov-1 sequences, regardless of whether they were single detection or co-detection, clustered together and no other branch was formed that was supported by bootstrap value ≥ 750 (Fig. 2). The identities for the HBov-1 complete genome were 99.4–100 %. The phylogenetic trees were similarly established for the NP1,

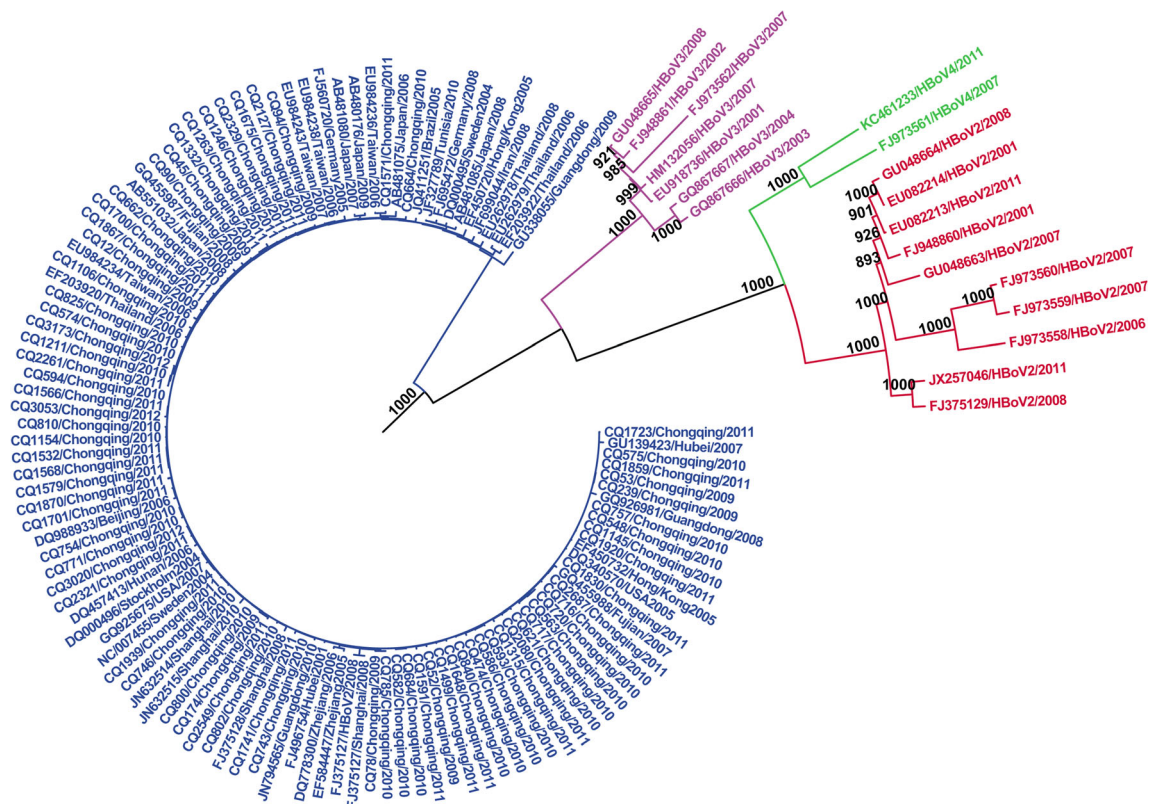


Fig. 2 Phylogenetic tree constructed based on 5,157 bp of the complete genome sequences of HBov-1 with the neighbor-joining method using CLC Main Workbench 5.5. The strain name in this study began with CQ.

HBov-1 is represented by the black line, HBov-2 by the blue line, HBov-3 by the green line, and HBov-4 by the red line

Table 2 Estimated mean evolutionary rates of HBoV-1 and the four genes

Gene	Mean evolutionary rate	95 % HPD
NS	8.43×10^{-5}	3.45×10^{-8} – 2.58×10^{-4}
NP	3.15×10^{-4}	$(1.05\text{--}5.22) \times 10^{-4}$
VP1	1.94×10^{-4}	$(0.79\text{--}2.99) \times 10^{-4}$
VP2	2.45×10^{-4}	$(1.13\text{--}3.71) \times 10^{-4}$
Complete genome	1.08×10^{-4}	$(0.40\text{--}1.86) \times 10^{-4}$

NS1, VP1, and VP2 genes (Supplemental Figure 1). The identities of HBoV-1 were 97.8–100 % for the NP1 gene, 99.6–100 % for NS1, 98.9–100 % for VP1, and 98.8–100 % for VP2. All the HBoV-1 viruses in the investigated localities were dominated by a single viral lineage, which had been dominant in most parts of China in recent years. Neither a spatial nor a temporal specific branch was formed based on the whole-genome analysis.

The overall evolutionary rate was estimated at 1.08×10^{-4} nucleotide substitutions per site per year (s/s/y) [95 % highest probability density (HPD): $(0.40\text{--}1.86) \times 10^{-4}$ s/s/y] based on the complete genome analysis. The highest evolutionary rate was derived from the NP gene, while the lowest was from the NS gene sequences (Table 2).

The SBP test showed no evidence of recombination for any of the four genes. Selective pressure analysis showed that all the ω -values were less than 1 (Table 3), indicating that these HBoV-1 genes were under negative selective pressure and the evolutionary process is shaped mainly by purifying selection. The NP gene had the largest global rate (0.23, 95 % CI: 0.15–0.33) among the four genes (range 0.08–0.23), suggesting that it is subject to greater selective pressure than the other three genes.

Site-by-site analysis of the full data set consisting of 206 HBoV-1 coding sequences identified only one site on the VP1 gene (codon site 40) undergoing significant positive selection and a number of negatively selected sites. A codon-based maximum likelihood reconstruction of the evolutionary history of codon site 40 under positive selection was carried out (Supplemental Figure 2). The result disclosed that the substitution has occurred mostly in the strains from Asia, especially

in China, suggesting that HBoV-1 strains from China undertake more selective pressure than those from other regions.

Discussion

In the current study, we have identified the clinical characteristics and temporal patterns of HBoV-1 in southeastern China through a 4-year surveillance. No role of HBoV-1 in severe pneumonia occurrence was observed, regardless of whether they were single detection or co-detection. In contrast, a significant association between HBoV and asthma was determined, suggesting a possible causal relationship. Based on the genetic analysis, HBoV-1 in southeastern China have similar mean evolutionary rates as other species of the family *Parvoviridae* and one positive selection site was also disclosed which differs from HBoV of other origins.

According to our results, the prevalence of HBoV-1 in pediatric ARTI patients is higher than those from other hospital-based studies [2, 6, 21, 22], whereas it was lower than that previously detected in persistently wheezing children [16]. HBoV-1 was commonly co-detected with a median rate of 42.5 %, and the most frequent co-detected viruses included rhinovirus, enteroviruses, and influenza [23]. We demonstrated a co-detection rate of 77.72 %, which is similar to the high proportion of co-detection as previously studied [24], yet significantly higher than those from other studies [9, 12]. These discrepancies could be explained by the inherent differences in the population subsets, detection methods, as well as the epidemiological variability relating to geographic origin and climatic factors.

Seasonal circulations of HBoV-1 vary, with most previous studies reporting HBoV-1 peak attained during the winter season [9, 22, 23]. Choi et al. found a higher frequency of HBoV-1 between May and July over a 5-year period among children with ARTI in Korea [25], which, interestingly, is consistent with our results displaying a summer peak. Although this temporal pattern coincided with the epidemic peaks of influenza circulation in southern China (<http://www.cnic.org.cn/chn/>), the single detection of HBoV also demonstrated a similar temporal pattern after excluding the

Table 3 Selective pressure analysis on the four genes of HBoV-1 by four methods

Gene	Model	Mean dN/dS (ω , 95 % CI)	Positively selected site			
			SLAC	FEL	IFEL	FUBAR
NS	HKY85	0.16 (0.11–0.22)	Null	Null	Null	Null
NP	HKY85	0.23 (0.15–0.33)	Null	Null	Null	Codon 79
VP1	TrN93	0.11 (0.08–0.13)	Codon 40	Codon 40	Codon 40	Codon 40
VP2	010023	0.08 (0.06–0.10)	Null	Null	Null	Null

effect from other respiratory pathogens, therefore indicating the actual seasonal pattern predominantly occurring in summer. An unexpected low peak in the winter of 2010 was demonstrated, probably caused by the extremely cold weather in Chongqing during the winter of that year (<http://www.cqmb.gov.cn/ecms/stny/nyqx/2011-03-11/3683.html>).

The relative importance of HBoV-1 as a causative pathogen for viral respiratory illnesses has not been determined, but it has been associated with respiratory diseases ranging from upper respiratory tract disease to bronchiolitis, and lower respiratory tract diseases, including pneumonia [11, 15, 26]. Vallet et al. found a high concordance between HBoV-1 detection and exacerbation of asthma requiring eventual hospitalization [27]. In our study, HBoV-1 detection was not associated with pneumonia. Instead, HBoV-1 tended to cause more upper respiratory tract infections, and its infection was also related with asthma exacerbation. Although a causal relationship between HBoV-1 infection and asthma cannot be concluded based on the statistical results, their significant association suggest the need for further investigation. Prospective longitudinal studies will hopefully increase our understanding of what, if any, role HBoV plays in asthma.

The epidemiology of the HBoV-1 circulation in China has been previously studied; however, the viral gene sequence data acquired from these studies were rare and insufficient to reveal any temporal or spatial characteristics or any evolutionary dynamics. Analysis of the genomic and epidemiological data of HBoV-1 from the current study, as well as those available from different localities, revealed limited heterogeneity, and high conservation was demonstrated from both spatial and temporal perspectives. Zehender et al. reported that the estimated mean evolutionary rate of the HBoV-1 VP2 gene was 8.6×10^{-4} s/s/y using 48 non-recombinant isolates [28], demonstrating that HBoV-1 was characterized by a rapid evolution. The rate identified in the current study is slower, which might be partially caused by the sample size of analyzed HBoV-1 sequences. A few of the previous studies explored the estimated evolutionary rates of parvoviruses in carnivore parvoviruses (7.9×10^{-5} – 1.7×10^{-4} s/s/y) [29] and human parvovirus B19 (1.1×10^{-4} – 2.6×10^{-4} s/s/y) [30]. The estimated mean evolutionary rates of the complete genome, VP1, VP2, and NS genes of HBoV-1 were similar to those of carnivore parvoviruses and human parvovirus B19. The NS gene was highly conservative among the four genes. Surprisingly, the NP gene showed a slightly higher substitution rate than the other genes.

The icosahedral capsid consists of two structural proteins, VP1 and VP2, which are identical except for a certain number of amino acids at the amino-terminal end of the VP1 protein, the so-called VP1 unique region (VP1U). A conserved phospholipase A2 (PLA2) motif, which resembles the catalytic motif of secreted PLA2 (sPLA2), has been identified in the

VP1U of most parvoviruses [31]. For HBoV-1, VP1 and VP2 have the same sequence of 542 aa at the C termini, while VP1 has an additional 129 aa peptide at the N-terminus. One study indicated that the VP1U of HBoV-1 also had sPLA2-like enzymatic activity, and these residues are crucial for its sPLA2-like activity [32]. Mutation of one of the amino acids (21Pro, 41His, 42Asp, or 63Asp) in the VP1U almost eliminated the sPLA2 activity of the HBoV-1 VP1U. Interestingly enough, one positive selection site (codon 40) was identified near codon 41 and 42 in the current study, which indicated a high probability of VP1U undergoing selective pressure. In spite of this finding, the associations between the positive selection site and sPLA2 activity or amplification of DNA need further investigation.

In conclusion, our study, for the first time, disclosed the epidemiological characteristics and genetic dynamics of HBoV-1 in southeastern China in recent years. The disclosure of the temporal pattern of HBoV infection provided the season when HBoV infection could be highly suspected and surveillance should be strengthened. The current data also demonstrated that HBoV was co-detected with a high prevalence; therefore, the sole presence of HBoV in patients should be interpreted carefully as to its causal role in causing disease. This information might help to further improve our understanding of HBoV-1 infection and guide better surveillance and control strategies in the future, especially among children.

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Conflict of interest The authors do not have any commercial or other association that might pose a conflict of interest. All authors read and approved the final manuscript.

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