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Clinical and genetic analysis of Human Bocavirus in children with lower respiratory tract infection in Taiwan

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ABSTRACT

Background: Human Bocavirus (HBoV) is a likely etiologic agent of acute respiratory disease in children. The prevalence of this virus has been studied in several sites worldwide. We conducted the first clinical and molecular study of HBoV in Taiwan at the Centers for Diseases Control, Taiwan.
Objectives: To investigate the genomic and epidemiologic profiles of HBoV infection in Taiwan.
Study design: Throat swabs or nasopharyngeal aspirates were obtained from hospitalized pediatric patients with acute lower respiratory tract infections. Specimens negative for other respiratory viruses by molecular screening were examined for HBoV.
Results: HBoV was the only virus detected in 30 (5.6%) of 531 samples. Of these positive cases, 56.7% were from children less than 2 years old. Two groups of HBoV co-circulated in Taiwan during the study. Results of evolutionary networks evaluation suggest that HBoV might have had an opportunity for interbreeding of viruses and genetic recombinations among the different genes.
Conclusion: HBoV may have circulated in Taiwan for some time and it appears to be one of the etiological agents responsible for lower respiratory tract infection in children.

1. Introduction

Human Bocavirus (HBoV), currently classified in the genus *Bocavirus* within the family *Parvoviridae,* was first described in 2005 after large-scale molecular screening of respiratory specimens for virus genome sequences led to its discovery.[1] The etiologic role of HBoV in the causation of respiratory illness is not clearly defined due to lack of viral propagation techniques in cell culture or animal models.[1] However, many studies have reported finding this virus associated with respiratory tract infections, especially in infants and young children.[1–5]. The virus has been reported worldwide,[6–11] with incidence rates determined by nucleic acid amplification ranging from 3 to 19%.[7,12–15] Recently, the virus has also been identified in blood and faecal samples.[12,16–19] Epidemiological data reported in most previous studies have been generated using the NP1 gene for HBoV detection. However, the complete coding sequence of this virus in the database is incomplete, and thus, the true prevalence of the virus may be slightly underestimated.[1] In order to better understand the genomic and epidemiological profiles of HBoV infection in Taiwan, we examined specimens from pediatric patients with lower respiratory tract infections (LRTIs). The genes of HBoV were determined using a newly designed set of conserved primers to both amplify and sequence. All experiments involving human material were approved by an ethical commission from the local partner university hospitals.

2. Materials and methods

2.1. Clinical specimens

Clinical specimens were obtained from pediatric patients hospitalized in Taiwan with acute LRTIs from October 2006 to

Abbreviations: HBoV, human bocavirus; LRTI, lower respiratory tract infection; RSV, respiratory syncytical virus; RT-PCR, reverse transcription polymerase chain reaction; ML, maximum likelihood; NJ, neighbor-joining; ORFs, open reading frames.

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Table 1
Primers for HBoV coding sequences analysis.

Gene target	Name	Primer sequences (5′–3′)	Position[a]	Amplicoon length (bp)
NS1	F161	5′-CCACGCTTGTGGTGAGTCTA-3′	161-180	1094
	R1254	5′-GCATGCCCAAGACTTGTTCT-3′	1254-1235	
	F1060	5′-GCCTAGCTGCGTCTTCTGTT-3′	1060-1079	1149
	R2208	5′-GTCTCAGGCTCGGTGTCTTC-3′	2208-2189	
NP-1	F2255	5′-CCAGCAAGTCCTCCAAACTC-3′	2255-2274	840
	R3094	5′-CGCGATCAGCGTTATTTACA-3′	3094-3075	

[a] Nucleotide position from GenBank database accession number NC_007455.

March 2007. Throat swabs or nasopharyngeal aspirates were obtained from patients upon hospital admission. Specimens were transported to a virology laboratory within 24 h. All samples selected for study were negative for influenza A and B viruses, parainfluenza virus types 1, 2, and 3, respiratory syncytical virus (RSV), and adenovirus by cell culture and immunofluorescence. All selected samples were also negative for human metapneumovirus and human coronaviruses 229E, OC43, and NL63 by reverse transcription polymerase chain reaction (RT-PCR).

2.2. Nucleic acid extraction and PCR

Viral DNA was extracted using either the QIAamp DNA Mini Kit (Qiagen, Santa Clara, CA) or an automated MagNA Pure LC instrument using the MagNA Pure LC Total Nucleic Acid Isolation kit

Table 2
Summary of clinical characteristics associated with Human Bocavirus (HBoV) infection in children with LRTIs.

Age (years)	Sex			Sample positive for HBoV(%)	Clinical manifestations in HBoV-positive patients		
	Female	Male	Total		Acute bronchitis	pneumonia	Duration of fever (days)
<2	63	105	168	17 (56.67)	4	13	1–7
3–4	63	65	128	7 (23.33)	0	7	2–8
5–6	62	63	125	4 (13.33)	1	3	3–4
7–8	40	27	67	2 (6.67)	0	2	6–8
9–10	12	17	29	0	0	0	0
11–12	1	1	2	0	0	0	0
13–14	3	5	8	0	0	0	0
>15	0	4	4	0	0	0	0
Total	244	287	531	30 (100)	5	25	

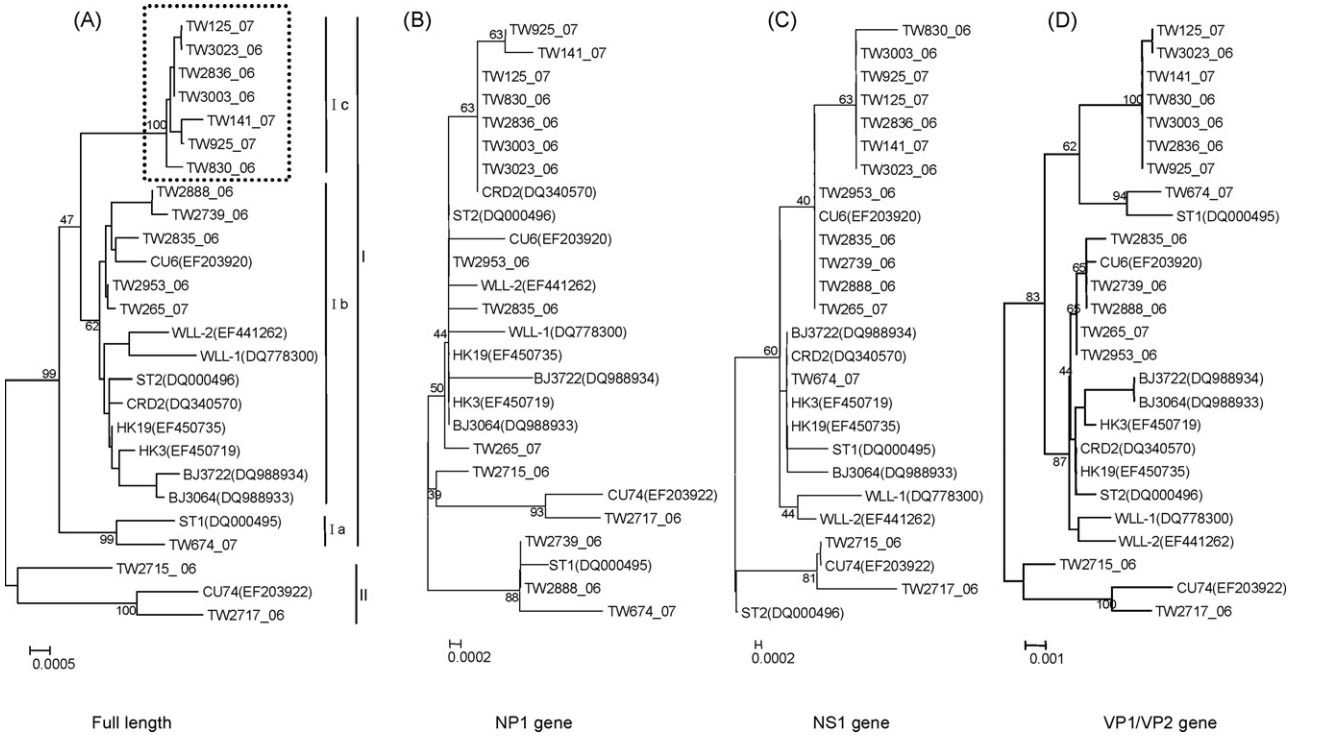

Fig. 1. Phylogenetic analysis of genetic evolution of full length, complete NP1, NS1, and VP1/VP2 gene sequences of 15 Human Bocavirus (HBoV) strains in Taiwan. The trees were inferred from full length (A), NP1 (B), NS1 (C), and VP1/VP2 (D) gene data by the neighbor-joining method and using bootstrap analysis ($n = 1000$) to determine the best-fitting tree. A total of 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. HBoV strains ST1 and ST2, from Sweden, were the 2 prototype strains.

Table 3
Amino acid variation of each coding regions of HBoV[a].

Group	Amino acid position	NS1					NP1				VP1/VP2													
		274	359	556	580	634	5	44	79	204	17	40	109	133/4	135/6	137/8	143/14	149/20	272/143	468/339	474/345	546/417	590/461
Ib	ST2(DQ000496)	T	H	A	D	D	N	S	S	E	R	L	A	T	I	D	V	T	A	S	N	N	S
	CDR2(DQ340570)																				S		
	HK3(EF450719)															N					S		
	BJ3064(DQ988933)														F	N	G				S		
	BJ3722(DQ988934)				N	N									F	N	G				S		
	CU6(EF203920)																				S		
	WLL-1(DQ778300)		R				S	N	N				V							T	S		
	WLL-2(EF441262)								N												S		
	HK19(EF450735)																				S		
	TW265_07																				S		
	TW2739_06																				S		
	TW2835_06																				S		
	TW2888_06																				S		
	TW2953_06																				S		
Ic	TW830_06									K											S		T
	TW2836_06																				S		T
	TW3003_06																				S		T
	TW3023_06																				S		T
	TW925_07																				S		T
	TW125_07																				S		T
	TW141_07																				S		T
Ia	ST1(DQ000495)																	A			S		T
	TW674_07			T							K								T		S		T
	TW2715_06																				S	H	
II	CU74(EF203922)	A										S		N							S	H	
	TW2717_06								N			S						N			S	H	

[a] Deduced amino acid difference in the HBoV, only locations where differences from the sequence of ST2 were observed are indicated.

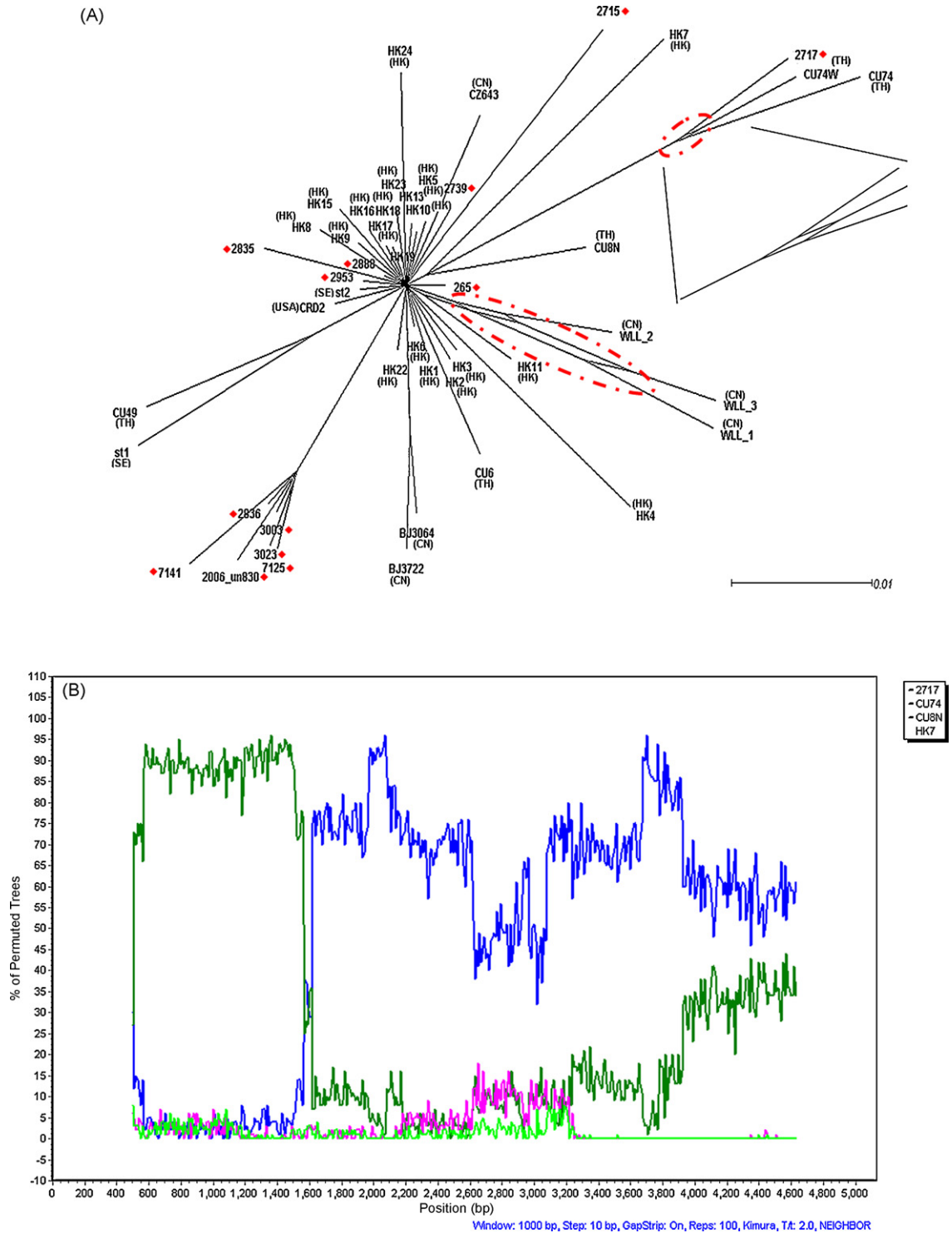

Fig. 2. Analysis of evolutionary networks of the complete full length gene from representative Taiwan's HBoV and its correlation with the appearance of other strains. Taiwanese viruses are marked with black diamond. Detect recombination of HBoV by split-decomposition method (A), the networks in black circles represent recombination happen to those isolates. Applied bootscan in Simplot software to detect the recombination point of HBoV 2717, CU74, and CU74W; and the most likely breakpoints were located between 1500 and 1600 bp of whole genome (B).

(Roche Diagnostics, Indianapolis, IN, USA). Extracts were stored at −80 °C. To reduce possible cross-contamination, the procedures from viral DNA extraction to PCR amplification were performed indifferent rooms on different days for each specimen. Real-time PCR reactions targeting the HBoV NP1 were performed according to previous publications[20,21] using an ABI Prism 7000 Real-time PCR system (Applied Biosystems, Forest City, CA, USA).

2.3. Complete genome sequencing and phylogentic analysis

The PCR product was purified using QIA quick spin columns (Qiagen, Valencia, CA, USA). Purified amplicons were cycle sequenced by using BigDye 3.1 Terminator cycle sequencing reagents, with reaction products resolved on an ABI Prism 3130XL DNA Analyzer (Applied Biosystems, Forest City, CA, USA). For phylogenetic

analysis, regions of the NS1, NP1, and VP1/VP2 genes were amplified and sequenced. Four primers were used to amplify and sequence the NS1 gene, and two primers were used to amplify and sequence the NP1 gene. Primer sequences are shown in Table 1. In addition, primers from a previous publication[22] were used to amplify and sequence VP1/VP2 genes. Complete coding sequences were determined according to a previous report[23]. Nucleotide sequence data were analyzed using Version 10.3 of the sequence analysis software package of the University of Wisconsin Genetic Group. Phylogeny construction and evaluation were performed with the maximum likelihood (ML) and neighbor-joining (NJ) methods in the Phylip software package (Version 3.66, University of Washington, Seattle, WA, USA)[24] and Bayesian analysis in the Mr. Bayes software (Version 3.1).[25,26] Empirical transition/transversion ratio was estimated by the TREE-PUZZEL software (Version 5.2)[27] to calculate evolutionary distances. The robustness of the NJ tree was statistically evaluated by bootstrap analysis with 1000 bootstrap samples. Since the ML method is already a statistically validated method (with a statistical evaluation of the branch length), no bootstrapping was done for this method. For Bayesian analysis, nucleotide substitution model was estimated by MrModeltest (Version 2.2).[28] Split decomposition in the Splits tree (Version 4.8, www.splitstree.org)[29] and BootScan in the SimPlot (Version 3.5.1, SCRoftware)[30] were used to detect evolutionary networks.

2.4. Accession numbers

The nucleotide sequences obtained in this study have been submitted to GenBank and have been assigned accession numbers EU984231–EU984245.

3. Results

The age range of all 531 patients studied was 1 month to 16 years. HBoV was the only virus isolated in 30 (5.6%) of the 531 cases. The medical records of the 30 patients with samples positive only for HBoV were reviewed. All 30 HBoV-positive cases were less than 8 years of age, and 17 (57%) were less than 2 years of age. Mean age (±S.D.) was 4.32 ± 2.98 years. The youngest HBoV-positive patient was 40 days of age at the time the respiratory specimen was obtained. The male:female ratio was 1.18:1. Twenty-five of the 30 HBoV-positive cases had pneumonia and 5 had acute bronchitis/bronchiolitis. Fever, cough and rhinorrhea were the most common symptoms. Days of fever ranged from 1 to 8 days (Table 2).

HBoV isolates obtained in this study were very similar, with highly conserved sequences among the different isolates. The 5.2 kb genome of HBoV contains 3 ORFs (open reading frames) encoding NS1, NP1, and VP1/VP2 genes. Phylogenetic analysis of these 3 ORFs in 15 HBoV strains did not reveal any genotypic differences between the strains from Taiwan and other countries (similarity 99–100%). Analysis of the nucleotide sequences encoding the complete genome (positions 1-5262) of Taiwanese strains showed that most of strains were genetically close to ST1 or ST2 (GenBank accession number DQ0000495 and DQ0000496, identified by Allander et al.[1], with the phylogenetic tree clearly divided into two major groups (I and II) (Fig. 1A). Group I could be further divided into three subgroups, the first comprised of ST1 and TW674_07viruses (Ia), the second comprised of ST2 viruses (Ib), and the third comprised of 7 Taiwanese strains (Ic). Group II was comprised of 3 strains (TW2715_06, TW2717_06, and CU74). These data demonstrate that at least 3 clusters of HBoV co-circulated in Taiwan during the study period. Amino acid sequences encoded by the NS1, NP1, VP1, and VP2 genes are shown in Table 3. All 7 Taiwanese strains in group Ic had amino acid changes at position S590T of VP1 numbering in comparison with sequence of ST2 (Table 3). Similarity plots of the HBoV TW2717_06 sequence with CU74 and CU74W consensus sequences suggested the presence of one point of crossover (Fig. 2B) with the most likely breakpoints located at 1500–1600 bp.

4. Discussion

This report is the first to evaluate HBoV infection in Taiwan. The percentage of HBoV-positive specimens among children with LRTIs was similar to that found in other studies of HBoV infection. In our study, HBoV was the only virus detected in nearly 10% of children less than 2 years old with LRTIs, a greater percentage than reported in other studies from Asia.[22,31–33] It is possible that there were additional coinfections not detected in our study since we did not test our respiratory samples for other potential pathogens such as rhinoviruses and enteroviruses. Our findings support the hypothesis that HBoV might play a role in the pathogenesis of LRTIs. Further studies are required to determine whether HBoV plays a causative role in LRTI or acts as an exacerbating factor that simple increases the severity of infections caused by other pathogens.

Phylogenetic analysis demonstrates that at least 2 groups and 3 clusters of one group of HBoV co-circulated during the study period. There was no temporal link between any of the groups as they were equally distributed throughout the study period. Each individual gene (Fig. 1) of HBoV was constructed and our results show that the greatest sequence variations occurred in the VP1/VP2 gene. Furthermore, our findings indicate that the two different groups of HBoV identified in Taiwan are genetically distinct. One is remarkably similar to the initial strains of HBoV identified in Sweden and the other one is similar to the strain (CU74) detected in Thailand. Likelihood-mapping analysis suggests that each ORF may have some "unresolved" phylogenetic signal, and that NS1 may not be the best gene with which to construct a phylogenetic tree. Phylogenetic trees constructed by NJ and ML methods showed a similar topology. A monophyletic group with longer branch length consisted only of Taiwanese HBoV isolates suggests that this virus might have circulated in Taiwan for a certain period (Fig. 1A). Moreover, computing the evolutionary networks from sequence data by applied splitstree software,[29] there appeared to be at least 2 networks among Asian strains (Fig. 2A). This finding suggests that the virus might have circulated simultaneously in several locations and that simultaneous infections with different strains might have occurred, resulting in an opportunity for interbreeding of virus and genetic recombination among the viral genes. It should be noted that recombinant HBoV has been reported; however, we are unaware of other HBoV mosaic genomes with a breakpoint pattern similar to the one presented here. This leads to speculation that if the recombinant variant continues to circulate, further genetic evolution may allow more efficient transmission in an exposed population.

Our study evaluated the phylogenetic relationships of the complete coding sequences of HBoV as well as each gene, and clearly demonstrates the value of analyzing the complete genetic composition in order to understand fully its molecular epidemiology. In conclusion, our data demonstrate that HBoV is circulating in Taiwan and suggest that HBoV might be involved in the pathogenesis of lower respiratory tract disease in children. Furthermore, our results indicate that genetically distinct HBoVs co-circulated in Taiwan during 2006–2007 and that recombinance among these viruses occurred contributing to the genetic diversity of the circulating strains. Importantly, data presented here provide evidence suggesting that HBoV may undergo gene recombination to evade host immunological response. Continued clinical and molecular surveillance in the future will be necessary to monitor the spread and epidemiological impact of HBoV.

Conflicts of interest

None of the authors had conflicts of interest.

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