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Human bocavirus (HBoV) in Thailand: Clinical manifestations in a hospitalized pediatric patient and molecular virus characterization

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Summary *Objective:* Human bocavirus (HBoV), a novel virus, which based on molecular analysis has been associated with respiratory tract diseases in infants and children have recently been studied worldwide. To determine prevalence, clinical features and perform phylogenetic analysis in HBoV infected Thai pediatric patients.
Methods: HBoV was detected from 302 nasopharyngeal (NP) suctions of pediatric patients with acute lower respiratory tract illness and sequenced applying molecular techniques.
Results: The incidence of HBoV infection in pediatric patients amounted to 6.62% with 40% co-infected with other respiratory viruses. There were no clinical specific manifestations for HBoV; however, fever and productive cough were commonly found. Generalized rales and wheezing were detected in most of the patients as well as perihilar infiltrates. The alignment and phylogenetic analysis of partial VP1 genes showed minor variations.
Conclusion: Our results indicated that HBoV can be detected in nasopharyngeal aspirate specimens from infants and children with acute lower respiratory tract illness.

Introduction

Acute respiratory tract infection is a major cause of pediatric morbidity and mortality worldwide. In most cases, viruses including influenza A and B, parainfluenza viruses, adenoviruses, respiratory syncytial virus (RSV), and human metapneumovirus (hMPV) are the causative agents. Recently, a new respiratory tract virus of the *Parvoviridae* family, Human Bocavirus (HBoV), has been discovered applying molecular analysis on pooled respiratory tract aspirations taken from children in Sweden. This virus is closely related to the bovine parvovirus and canine minute virus, which have been classified as members of the genus

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Bocavirus. The virus comprises two major open reading frames (ORFs) encoding a nonstructural protein (NS1) and at least two capsid proteins (VP1 and VP2), respectively. Moreover, the HBoV genome also contains a third middle ORF encoding a nonstructural protein (NP1) of unknown function.[1] The most conserved region of this virus is the NS1 and NP1 gene whereas the VP1/VP2 gene constitutes the variable region.[2] The induction of respiratory illness by HBoV is not clearly defined due to lack of propagation techniques in cell culture or animal models.[1] However, many studies have reported this virus infection to be associated with acute respiratory illness.[1,3,4] Upon discovery of HBoV in respiratory pools, its global prevalence has been reported to range from 1.5% to 19% and co-infection with other viruses was commonly found.[1,3–24] Moreover, in few studies were shown negative results for HBoV infection in nasopharyngeal (NP) swabs from healthy volunteers.[20,25,26] Additional epidemiological and clinical investigation will be essential in order to elucidate what exactly engenders HBoV related illness. Therefore, in the present study we applied polymerase chain reaction to detect HBoV from NP suctions collected from infants or children who had been admitted with respiratory tract illness.

Materials and methods

Clinical samples

Nasopharyngeal suction specimens were collected from 302 individual infants or children (age range: 5 days to 14 years) who were admitted and diagnosed as having acute lower respiratory illness during the period February 14, 2006 to February 28, 2007. All of the clinical samples were provided by the Department of Pediatrics, King Chulalongkorn Memorial Hospital, Thailand. Nasopharyngeal suction samples were collected in transport medium with antibiotics (0.5% BSA, penicillin G (2×10^6 U/L), streptomycin 200 mg/L) and stored at −70 °C until tested. The study was conducted after receiving approval by the Ethics Committee of the Faculty of Medicines, Chulalongkorn University. Prior to enrolment, all participating parent gave their written informed consent.

DNA and RNA extraction

DNA and RNA were extracted from 150 μl of NP suction using TRI REAGENT® LS (Molecular Research Center Inc., Cincinnati, OH) and solubilized in 20 μl of 8 mM NaOH or 12 μl of DepC treated water for DNA or RNA, respectively.

Reverse transcription

Reverse transcription (RT) was performed at 37 °C for 2 h using 200 units of M-MLV reverse transcriptase (Promega, Madison, WI), 5 μl of 5× M-MLV reaction buffer (Promega), 5 μl of 10 mM dNTP (Promega), 25 units of RNasin® Ribonuclease Inhibitor (Promega) and 0.5 μg/μl of Random Primer (Promega), 12 μl of RNA heating to 70 °C for 5 min then cooling on ice, and adding nuclease-free water to a final volume of 25 μl.

Detection of HBoV and other respiratory viruses

For HBoV detection we amplified the NP1 gene by conventional PCR modified from a previous study.[1] The reaction mixture contained 2 μl DNA, 0.5 μM 188F primer and 0.5 μM 542R primer, 10 μl 2.5× Eppendorf masterMix (Eppendorf, Hamburg, Germany), and nuclease-free water to a final volume of 25 μl. The amplification reaction was performed in a thermocycler (Eppendorf) under the following conditions: Initial denaturation at 94 °C for 3 min, followed by 35 amplification cycles consisting of 94 °C for 30 s (denaturation), 55 °C for 30 s (primer annealing), and 72 °C for 1 min (extension), and concluded by a final extension step at 72 °C for 7 min. Another set of primers specific for the VP1 gene, VPF2 forward primer (5′-TTCAGAATGGT CACCTCTACA-3′: nt 3639—3659) and VPR2 reverse primer (5′-CTGTGCTTCCGTTTTGTCTTA-3′: nt 4286—4266), were used in a separate PCR reaction to exclude false positives. After 2% agarose gel electrophoresis stained with ethidium bromide, the expected products of 354 and 648 bp representing the NP1 and VP1 gene, respectively, were visualized on a UV transilluminator.

Influenza A virus detection was performed by conventional PCR using 1 μl of cDNA, 0.5 μM of FluA_M_F: 5′-RGGCCCCCTCAAAGCCGA-3′ (nt 76—93), 0.5 μM of FluA_M_R: 5′-ACTGGGCACGGTGAGYGT-3′ (nt 235—218), 10 μl of 2.5× Eppendorf masterMix, and nuclease-free water to a final volume of 25 μl. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of each sample was amplified in one additional reaction mixture of identical volume with primers GAPDH_F: 5′-GTG AAGGTCGGAGTCAACGG-3′ (nt 112—131) and GAPDH_R: 5′-GTTGTCATGGATGACCTTGGC-3′ (nt 603—583) at a 0.5 μM concentration, each. The amplification reaction was performed in a thermocycler (Eppendorf) under the following conditions: Initial denaturation at 94 °C for 3 min, followed by 40 amplification cycles consisting of 94 °C for 30 s (denaturation), 55 °C for 30 s (primer annealing), and 72 °C for 1 min (extension), and concluded by a final extension step at 72 °C for 7 min. After 2% agarose gel electrophoresis, the expected products were influenza A virus (160 bp) and the housekeeping gene (492 bp).

Parainfluenza virus was detected by real-time PCR using SYBR green I carried out in a Rotor-Gene 3000 (Corbett Research, New South Wales, Australia). The reaction mixture contained 1 μl of DNA sample, 10 μl 2.5× Eppendorf masterMix, 0.5 μM ParaF: 5′-GCTAAATACTGTCTTMAH TGGAGAT-3′ (nt 11,254—11,278), 0.5 μM ParaR: 5′-GTAAGG ATCACCWACATADAWTGTA-3′ (nt 11,392—11,370), 2 μl 1× SYBR Green and nuclease-free water to a final volume of 20 μl. The amplification reaction consisted of a preincubation step at 95 °C for 3 min followed by 35 cycles of amplification including 95 °C for 15 s, 55 °C for 15 s and 72 °C for 30 s. The fluorescent signal was detected once per cycle upon completion of the extension step. After amplification, melting curve analysis was performed by heating to 95 °C then cooling to 60 °C for 15 s, followed by a temperature increase to 95 °C, while continuously collecting the fluorescent signal data.

Adenovirus, influenza B virus, respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) were detected

by the method described by Krafft et al.,[27] Chi et al.,[28] Samransamruajkit et al.[29] and Thanasugarn et al.,[30] respectively.

Molecular characterization and phylogenetic analysis

All HBoV positive samples were subjected to VP1 gene sequencing. The partial VP1 gene at the 5′ end of HBoV was amplified into two segments with two primer sets, VPF1 (5′-GATAACTGACGAGGAAATGCT-3′: nt 3009—3029) and VPR1 (5′-AGTATGTCCATGGAGTTGTGA-3′: nt 3731—3711) for the first segment and VPF2 and VPR2 for the second. The expected product sizes after 2% agarose electrophoresis were 723 and 648 bp, respectively. PCR conditions have been described elsewhere.[2] The PCR products were purified using the Perfectprep Gel Cleanup kit (Eppendorf). DNA sequencing was performed using the Gene Amp PCR system 9600 (Perkin—Elmer, Boston, MA). The sequencing products were subjected to a Perkin Elmer 310 Sequencer (Perkin—Elmer) for subsequent sequence analysis. The DNA sequences were analyzed with the BLAST (http://www.ncbi.nlm.gov/BLAST) program and the phylogenetic analyses and genetic comparisons between HBoV strains were performed using the Molecular Evolutionary Genetics Analysis (MEGA) version 3.1 program.

Results

Detection of HBoV and other viruses

We applied PCR and RT—PCR to detect HBoV, other respiratory viruses and GAPDH. Of 302 specimens, 20 (6.62%) were positive for HBoV. All specimens had been collected throughout the year and plotting of seasonal HBoV detection was shown in Fig. 1. Among the 302 specimens we also detected co-infection with other respiratory viruses such as RSV in 48 (15.89%) samples, influenza A virus in 33 (10.92%) samples, hMPV in 28 (9.27%) samples, adenovirus in 18 (5.9%) samples, parainfluenza in 14 (4.63%) samples, and influenza B virus in 1 (0.33%) sample. HBoV-positive samples co-infected with other respiratory viruses comprised altogether 40% (Table 1).

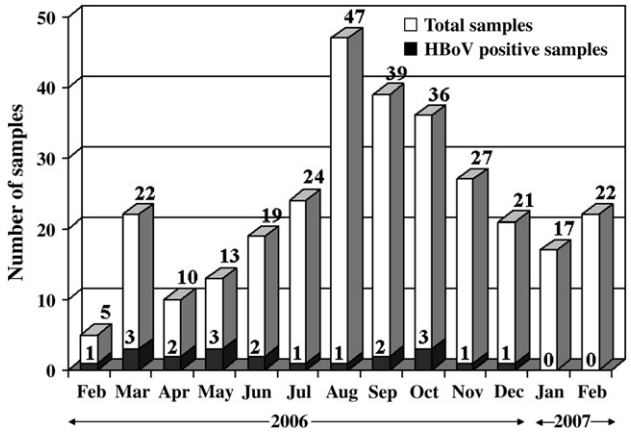

Figure 1 Detection of HBoV from 14 February, 2006 to 28 February, 2007.

Clinical manifestations

The clinical manifestations of HBoV infected patients are summarized in Table 1. The majority of infants positive for HBoV were male (14/20), between 4 and 36 months old (median age 12 months). On average, they were hospitalized for 6.15 days (range 2—22 days). The most common clinical symptom in all HBoV infected infants was fever with a mean duration of 3.47 days and other common symptoms such as runny nose (55%) and productive cough (50%) were found. Generalized rales were the most common (70%) and wheezing the second most common lung signs (35%). Acute bronchiolitis was diagnosed in 9 out of 20, and 11 samples had viral pneumonia. Perihilar infiltration was ubiquitously present on the chest X-ray of HBoV positive patients (except for CU6 and CU15 where no chest X-ray result was available) (data not shown). Additional diseases of some patients with HBoV infection included congenital heart disease (CU33 and CU74), chronic lung disease (CU 31 and CU171), asthma (CU71) and cow milk protein sensitive enteropathy (CU157).

Molecular characterization and phylogenetic analysis of the HBoV VP1 gene

The nucleotide sequences obtained from this study have been submitted to the GenBank database under accession numbers: EF690641—EF690657 (Table 1) except for CU6 (EF203920), CU49 (EF203921) and CU74 (EF203922) that were subjected to analysis for complete coding sequences as described elsewhere.[2] The 5′ terminal 1182 base pairs (bp) of the VP1 gene were sequenced in all HBoV positive samples for phylogenetic analysis. Other VP1 sequences from several areas were available at the GenBank database and used for phylogenetic analysis including sequences from China: DQ778300, EF441262, EF584447, DQ457413, DQ987595, DQ987596, DQ677523, DQ494203, DQ457415, DQ988933, DQ988934, DQ987597, DQ987598 and DQ494201, Japan: EF035488, USA: DQ340570, and Sweden: DQ000495 and NC_007455. The constructed phylogenetic trees of the VP gene (nt 2986—4167) are shown in Fig. 2. Alignment of these partial VP1 genes showed minor variations in that the percent identity with the st1 Swedish prototype strain ranged from 98.6% to 99.5%. A phylogenetic tree of the HBoV VP gene was constructed based on neighbor-joining (NJ) trees. Confidence values for the tree topologies were evaluated by bootstrap analysis of 1000 pseudo-replicate datasets that showed the low genetic diversity.

Discussion

In the past few years, applying molecular techniques has led to the discovery of HBoV as a novel virus apparently associated with respiratory tract illness in humans.[1] However, due to lack of suitable culture systems and animal models to propagate the virus previous studies could not meet with Koch's postulates in order to clarify that HBoV can cause respiratory illness. It can be concluded from

Table 1 Clinical manifestations in HBoV positive patients

Samples No.	Accession no.	Sex	Age (months)	Duration of hospitalization (days)	Diagnosis[a]	Clinical manifestations	Virus detection[b]					
							Adeno	Paraflu	RSV	Flu A	Flu B	hMPV
CU6[2]	EF203920	M	36	2	VP	High fever for 2 days, dry cough, and crepitation both lungs	+	+	+	−	−	−
CU15	EF690641	M	24	4	AB	Low grade fever for 4 days, clear runny nose, diarrhea, and rhonchi	−	+	−	−	−	−
CU23	EF690642	F	12	2	AB	Fever for 7 days, clear runny nose, wheezing, and rales	−	−	−	+	−	−
CU25	EF690643	M	12	10	AB	Fever for 4 days, intractable cough, diarrhea, wheezing, rales, and subcostal retraction	+	−	−	−	−	−
CU31	EF690644	M	7	8	VP	Fever for 7 days, runny nose, expiratory rhonchi, and rales	−	−	−	−	−	−
CU33	EF690645	M	12	7	AB	Fever for 5 days, productive cough, breathing difficulty, bilateral rales, and expiratory wheezing	−	−	−	−	−	−
CU40	EF690646	M	4	6	AB	High fever for 1 day, severe productive cough, and expiratory wheezing	−	−	−	−	−	−
CU43	EF690647	M	7	16	AB	Fever for 2 days, clear runny nose, and medium rales	−	−	−	−	−	−
CU49[2]	EF203921	F	24	5	VP	Low fever for 3 days, breathing difficulty, and rales	−	−	−	−	−	−
CU55	EF690648	M	24	4	VP	Fever for 2 days, runny nose, cough, and rales	−	−	−	−	−	−
CU71	EF690649	F	24	5	VP	Fever for 3 days, dry cough, runny nose, and crepitation both lungs	−	+	−	−	−	−
CU74[2]	EF203922	M	12	7	VP	Low grade fever, clear runny nose, diarrhea, and medium rales	−	−	−	−	−	−
CU123	EF690650	M	12	3	VP	Fever for 3 days, clear runny nose, and rales	−	−	+	−	−	−
CU157	EF690651	M	11	22	VP	Fever for 7 days, intractable cough, fine crepitations, and hypovolemic shock	−	−	−	−	−	+
CU171	EF690652	F	6	5	VP	Fever for 1 day, runny nose, and rales	−	−	−	−	−	−
CU194	EF690653	M	12	2	VP	Fever 3 days, productive cough, breathing difficulty, bilateral rales, and expiratory wheezing	−	−	−	−	−	−
CU205	EF690654	F	12	2	AB	High fever for 2 days, severe productive cough, and expiratory wheezing	−	−	−	−	−	−
CU218	EF690655	F	36	4	AB	Fever for 2 days, clear runny nose, diarrhea, and medium rales	−	−	+	−	−	+
CU253	EF690656	M	12	4	AB	Low fever for 1 day, breathing difficulty, wheezing, and rales	−	−	−	−	−	−
CU256	EF690657	M	12	5	VP	Fever for 7 days, runny nose, diarrhea, and rales	−	−	−	−	−	−

Adeno, adenovirus; Paraflu, parainfluenza virus; Flu A, influenza A virus; Flu B, influenza B virus; RSV, respiratory syncytial virus and hMPV, human metapneumovirus.

[a] Physician's diagnosis: VP, viral pneumonia; AB, acute bronchiolitis.

[b] Other respiratory viruses detected by PCR or RT—PCR.

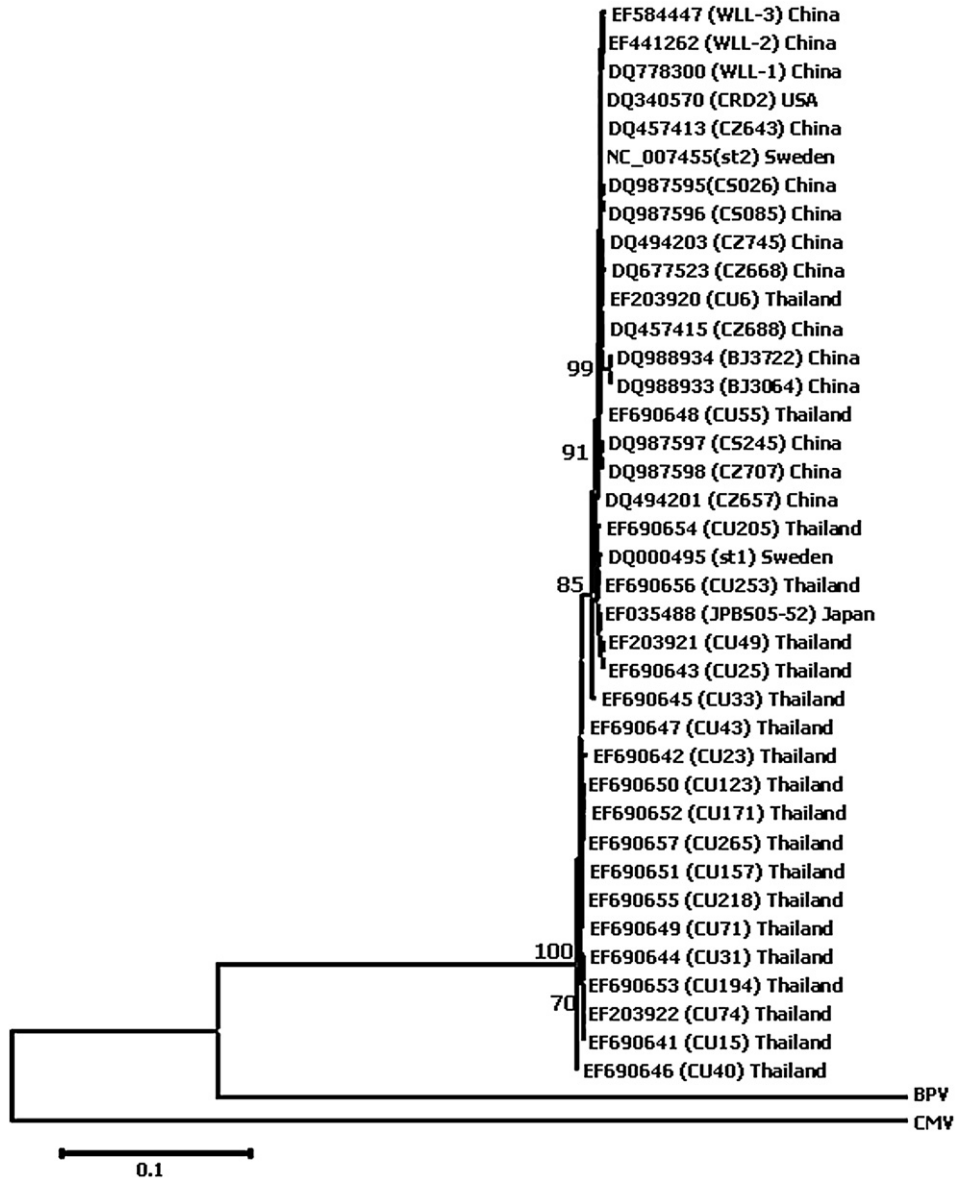

Figure 2 Phylogenetic analysis of the VP gene (nt 2986—4167) of HBoV in which the other members of *Bocavirus* genus, Bovine parvovirus (BPV) and Canine minute virus (CMV) were served as the outgroup sequences. The tree was created by Neighbor-Joining method and bootstrapped with 1000 replicates. The bootstrap numbers were given for each node. Only bootstrap values above 70 are shown.

epidemiology data that HBoV has been distributed throughout every continent with a different incidence rate (1.5% to 19%). In this study, the NP suctions had been collected from pediatric patients hospitalized with acute lower respiratory tract illness. HBoV has been detected in 20 (6.62%) out of 302 NP suction samples, whereas the previous study in the rural area of Thailand had the prevalence of 4.5%.[18] Co-infection with other respiratory viruses was found in 40% of the samples tested. RSV and parainfluenza virus were frequently detected as co-infecting viruses. However, some of the respiratory viruses were not included in this study, for examples; rhinoviruses and coronaviruses. Therefore, the percentage of co-infection may be higher than this report. Thailand has a tropical climate country and seasonal detection of HBoV can not be deduced from the few (1—3) HBoV-positive samples found in each month. RSV and hMPV were shown the seasonal distribution that peaked in July to September which correlated to the number of samples collection (data not shown). Moreover, the study of Mannig et al. reported a similarity in seasonal appearance between HBoV and RSV[12] whereas Weissbrich et al. did not.[16] The clinical features commonly found in HBoV positive patients were fever and productive cough. Bilateral rales and wheezing were among the most common abnormal lung signs observed and almost equally found in these patients (Table 1). These findings might indicate both lung parenchyma and airway involvement by this pathogen. Furthermore, the detection of this virus in specimens aspirated from the nasopharynx together with the significant lower respiratory tract illness indicated the lung pathology in these patients.

In conclusion, we detected HBoV infection in 6.62% and co-infection with other respiratory viruses in 40% of the NP suctions obtained from infants and children with acute lower respiratory tract illness with non-specific clinical features and age distribution. Seasonal appearance of HBoV was not significant. Based on phylogenetic analysis of the VP1 gene, the low genetic diversity was defined. The present study has investigated associations of HBoV with clinical manifestations and performed genome analysis but in order to completely describe this novel virus, further studies on serology, tissue and animal culture systems or clinical manifestation inductions will be required.

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