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Detection of Human Bocavirus Genotype 1 (HBoV1) in Children with Acute Asthma and Wheezing Attacks

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ABSTRACT

Background: *The sole pathogenic role of human bocaviruses, which have recently been discovered and known to cause acute respiratory diseases in children, has not yet been completely understood.* ***Objective:*** *To demonstrate the etiological role of Human Bocavirus alone in children with acute asthma and wheezing attacks.* ***Methods:*** *Nasopharyngeal aspirate (NPA) specimens collected from children who were hospitalized with severe acute asthma and wheezing attacks were tested for Human Bocavirus (HBoV) by PCR with specific primers to HBoV. In addition to, DNA sequence analysis and Phylogenetic analysis of the specific HBoV amplicons were performed.* ***Results:*** *In PCR with primers targeting the HBoV VP1/VP2 and NS1 genes, single and very sharp amplication bands of the expected length of 0.57Kb and 0.35Kb revealized from 3 of 6 NPA specimens. DNA sequence analysis and Phylogenetic analysis of the specific amplicons of HBoV VP1/VP2 and NS1 genes revealed that the virus detected by PCR was HBoV genotype 1 with 98.3~99.9% similarity.* ***Conclusions:*** *The obtained result revealed that our study provides strong support that HBoV can cause severe acute asthma and wheezing attacks in children in the absence of viral and bacterial co-infections.*

Keywords: Human Bocavirus (HBoV), Asthma, Wheezing, PCR, DNA Sequence Analysis, Phylogenetic Analysis.

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1. INTRODUCTION

The human bocavirus (HBoV), a member of the Bocavirus genus of the Parvoviridae family was firstly discovered in 2005 by Swedish researcher Allander et al.[1)] in Sweden, and were classified into four genotypes HBoV1–4 based on nucleotide divergence of the nonstructural protein, NS1 or the structural capsid protein, VP1.[1, 3, 4, 5, 9, 19)] . HBoV1 is one of the most commonly detected respiratory

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viruse, and is the most importance genotype of HBoVs. HBoV1 is more frequently detected in young children (<3 years of age) than in older children or adults. HBoV1 causes a variety of mild to severe upper or lower respiratory tract infections (LRTIs) in children (mainly between 6 months and 5-year-old).[7, 16, 17, 18, 25, 26, 28)]

Recently, several researchers frequently reported that HBoV1 is an important causative agent which causes various clinical symptoms and syndrome of the typical respiratory diseases such as fever, cough, hypoxia, respiratory distress, wheezing, asthma in young children.[2, 6, 12, 20)] In addition to, some studies on HBoV1 have shown that it may be a causal agent of severe lower respiratory tract infections such as bronchiolitis, bronchitis, Pneumonia in children.[11, 13, 22, 23)] Also, some authors indicated that HBoV1 infection may be a trigger for exacerbation of asthma in young infants, especially ＜2 years of age.[8, 10, 21, 24)]

However, the pathogenic role of HBoV1 as a sole causative factor in an acute respiratory tract infections has been often questioned by presence of HBoV1 together with another respiratory virus in same samples (up to 90%) and detection of bocavirus also in asymptomatic children (up to 44%).[7, 10, 14, 15, 27)]

In this study, we report that we verified the association between HBoV1 infection and the occurrance of the severe acute respiratory infections in children such as acute asthma and wheezing attacks with by PCR and DNA sequencing.

2. MATERIALS AND METHODS

2.1. Patients and Clinical Specimens

Between November 2024 and January 2025, NPA specimens (n=6) were collected from children (＜ 3 years of age) hospitalized with acute asthma and wheezing attacks at the Pediatric Hospital in Pyongyang University of Medical Sciences, placed in a viral transfer medium, immediately transferred to the laboratory, and stored at -80℃ before testing.

2.2. Detection of HBoV1 by PCR

PCR for the detection of HBoV was performed using specific primers previously described targeting the viral capsid protein (VP1/VP2) gene and non-structural protein (NS1) gene of HBoV [25, 26]. HBoV-1F (5’-GGCTCCTGCTCTAGGAAATAAAGAG-3’) and HBoV-1R (5’-CCTGCTGTTAGGTCGTTGTTGTATGT-3’) primers were targeted to the VP1/VP2 gene and the expected product sizes were 576 bp. HBoV-2F (5’-TGGCTACACGTCCTTTTGAACC-3’) and HBoV-2R (5’-GACTTCGTTATCTAGGGTTGCG-3’) primers were targeted to the NS1 gene and the expected product sizes were 347 bp. The primers used for PCR were synthesized by Sangon, Biotech Co, Ltd, China.

Total viral nucleic acid was extracted from 140 μL of NPA specimens using a QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. This kit is useful for the simultaneous extraction of viral RNA and DNA.

Subsequently, we used the HotStart PCR kit (Qiagen, Germany) and one-step RT-PCR kit (Qiagen, Germany) to detect and screen the presence of HBoV and other 6 respiratory viruses, namely, human parainfluenza virus 1 (HPIV-1), human parainfluenza virus 3 (HPIV-3), influenza A viruses (A(H1N1) and A(H3N2)), human respiratory syncytial virus (HRSV), and human metapneumovirus (HMPV).

Briefly, each PCR mix for HBoV contained 0.4 μM of each primer, 0.5 μL of 10 mM dNTPs, 2.0 μL of 10× Qiagen PCR buffer (Qiagen), 1.0 U of Qiagen HotStart Taq polymerase (Qiagen), and 2 μL of nucleic acid extract in a final volume of 20 μL. PCR was performed on an DNA Engine thermal cycler (Bio-Rad, Gladesville, Australia) with the following thermocycling conditions; a 12 min incubation at 95°C, followed by 40 cycles of 94°C for 30 sec, 48~56°C for 30 sec, and 72°C

for 60 sec, followed by a final extension of 72°C for 5 min.

In addition to, each RT-PCR mix comprised 4 µL of 5× QIAGEN RT-PCR buffer, 0.5 µL of forward and reverse primers (10 µM), 1 µL of Enzyme Mixture, 0.5 µL of dNTPs, 0.5 µL of RNase inhibitor, 2 µL of template RNA, and 11 µL of ddH_2O in a final volume of 20 µL. The RT-PCR conditions were 50°C for 30 minutes and 95°C for 15 minutes, followed by 40 cycles of 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 1 minutes, followed by a final extension of 72°C for 5 min. PCR and RT-PCR products were visualized by electrophoresis on 1.5% agarose gel with ethidium bromide staining.

2.3. DNA sequencing of PCR product for HBoV and Phylogenetic analysis

The amplified products of HBoV VP1/VP2 gene and NS1 gene were purified using a DNA Gel Extraction kit (MicroDNA Gel Extraction Kit, Sangon Biotech) and sequenced in forward and reverse directions using a BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer Applied Biosystems, Tokyo, Japan) with an ABI Prism 3500 Genetic Analyzer DNA sequencer (Perkin-Elmer Applied Biosystems).

Nucleotide sequences of amplified HBoV VP1/VP2 gene and NS1 gene products were aligned by using CLUSTAL W software. All nucleotide sequences were compared to those of reference strains available in the NCBI GenBank database using the BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phylogenetic and molecular evolutionary analysis was performed by the neighbor-joining algorithm and Kimura two-parameter model, supported by bootstrapping with 1000 replicates in MEGA software (ver. 7.0). Phylogenetic trees of the partial HBoV VP1/VP2 gene and NS1 gene were constructed by the clustal method using Megalign (Lacergene, DNA STAR, Inc., Madison, WI, USA).

2.4. Coinfections with other respiratory viruses

All NPA specimens with positive HBoV DNA in PCR were tested for coinfections with other respiratory viruses such as HPIV-1, HPIV-3, IFV-A, IFV-B, RSV, and HMPV using specific primers respetively.

2.5. Analysis for Clinical features and outcomes of patients

Basic conditions and Clinical features of the patients at admission such as sex, age, body weight, body temperature, white blood count, major symptoms, basic status in birth (gestational weeks), diagnosis in hospitalization, chest X-ray imagings, illness severity, and length of hospitalization were recorded and analyzed.

3. RESULTS AND DISCUSSIONS

3.1. Detection of HBoV

3.1.1. Detection of HBoV by VP1/VP2 gene specific primer

Firstly, we performed PCR with primer pair targeting the VP1/VP2 gene of HBoV in condition of 48°C annealing and 6 nucleic acid extracts extracted from NPA specimens of patients with asthma/wheezing as template (Fig. 1).

As a result, sharp amplication bands of the expected length of 0.57Kb revealized from 3 of 6 nucleic acid extracts extracted from NPA specimens of patients with asthma/wheezing, although several nonspecific bands were coexisted in PCR products.

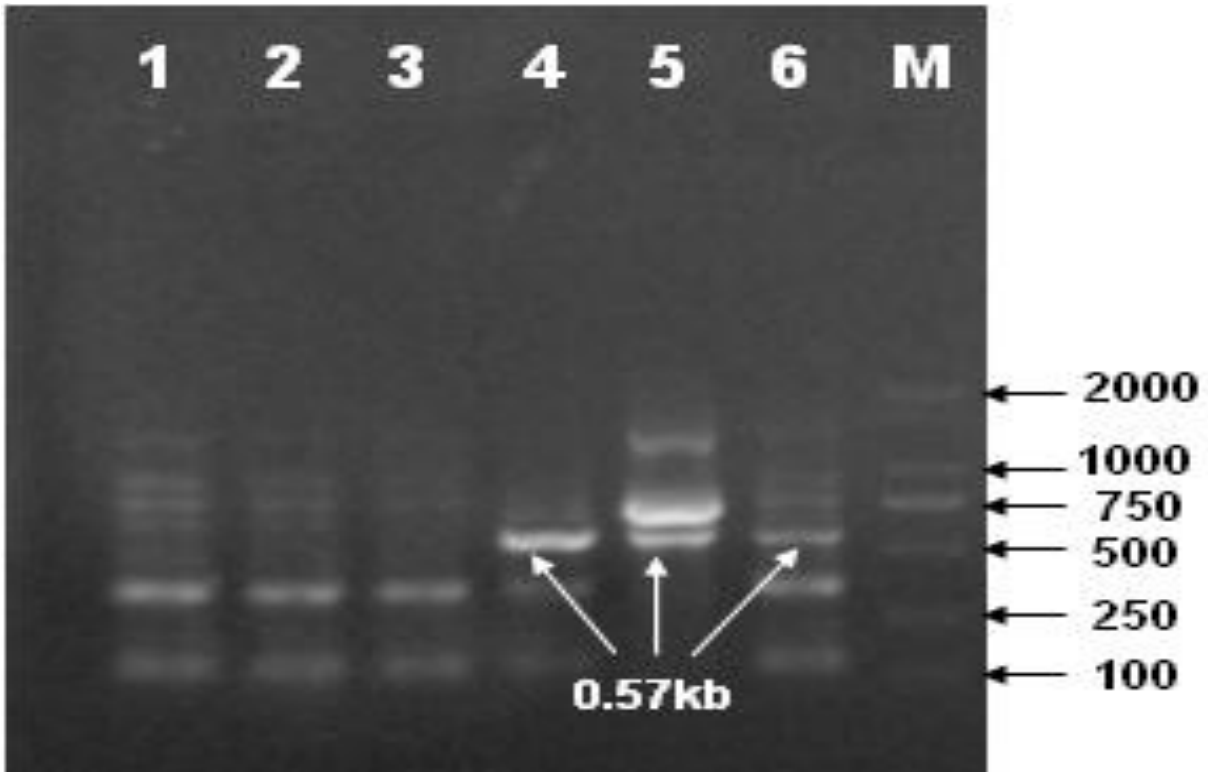

Fig. 1. PCR for detection of HBoV by VP1/VP2 gene specific primer in condition of 48°C annealing : lane 1~6: PCR products specific to HBoV1 VP1/VP2 gene , lane M: DL2000 molecular marker (1.5% AGE).

And then, PCR for HBoV detection conducted under optimal amount of the template (1~2ng for 10 μL of PCR) and annealing conditions (56 °C) provided a single amplification bands without any nonspecific bands (Fig. 2).

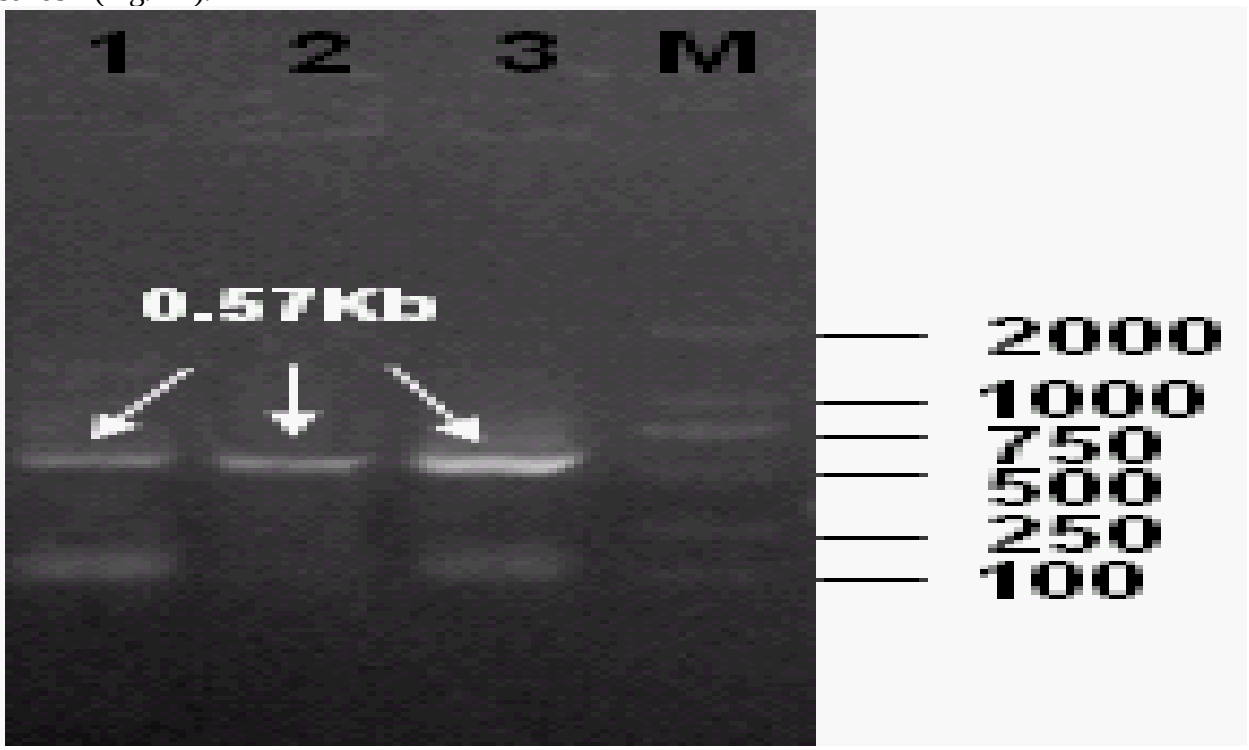

Fig. 2. PCR for detection of HBoV by VP1/VP2 gene specific primer in condition of 56°C annealing : lane 1~3: PCR products specific to HBoV VP1/VP2 gene , lane M: DL2000 molecular marker (1.5% AGE). 3.1.2. Detection of HBoV by NS1 gene specific primer

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Secondary, we conducted PCR to 6 nucleic acid extracts including 3 nucleic acid extracts positive in HBoV VP1/VP2 gene-based PCR with primer pair targeting the NS1 gene of HBoV (Fig. 3).

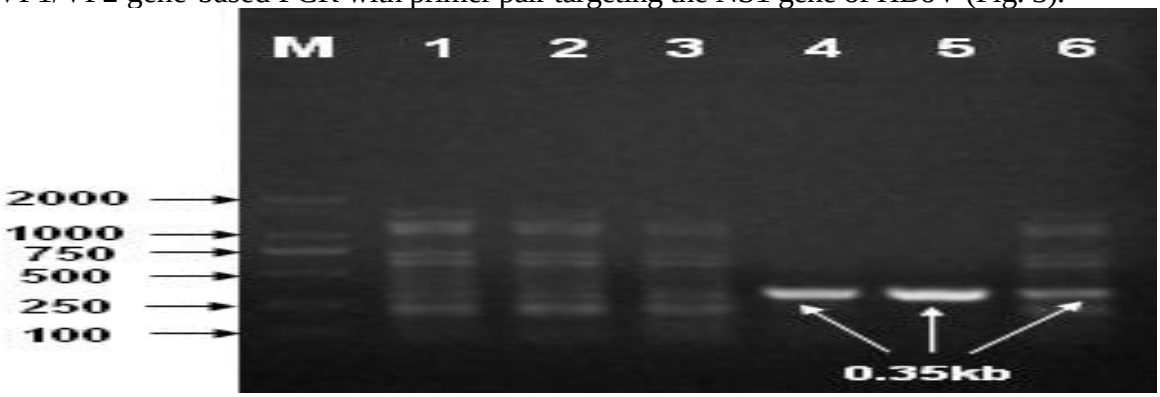

Fig. 3. PCR for detection of HBoV by NS1 gene specific primer in condition of 52°C annealing : lane 1~6: PCR products specific to HBoV NS1 gene , lane M: DL2000 molecular marker (1.5% AGE) .

As a result, single and very sharp amplication bands of the expected length of 0.35Kb revealized from 2 of 3 nucleic acid extracts positive in HBoV VP1/VP2 gene-based PCR, although another nucleic acid extract showed a specific amplication band of the expected length (0.35Kb) with several nonspecific bands in PCR products.

3.2. Identification of HBoV by DNA Sequencing and Phylogenetic analysis.

3.2.1. Identification of HBoV based on VP1/VP2 gene specific amplication bands

The products (0.57kb) from positive HBoV VP1/VP2 gene specific PCR were purified and sequenced in forward directions using Big Dye terminator chemistry and the ABI Prism 3500 Genetic Analyzer DNA sequencer (Perkin-Elmer Applied Biosystems). As a result, 373 nucleotide sequences were analyzied from the amplified products (0.57kb) of HBoV VP1/VP2 gene. The analyzied

AAATACTTTTACTTTGCTAACTCAAATAAAGGTGCAAAAAAAACAAAAAAAAGTGAACCTAA
ACCAGGAACCTCAAAAATGTCTGACACTGACATTCAAGACCAACAACCTGATACTGTGGACGCA
CCACAAAACACCTCAGGGGGAGGAACAGGAAGTATTGGAGGAGGAAAAGGATCTGGTGTGGGG
ATTTCCACTGGAGGGTGGGTCGGAGGTTCTCACTTTTCAGACAAATATGTGGTTACTAAAAACA
CAAGACAATTTATAACCACAATTCAGAATGGTCACCTCTACAAAACAGAGGCCTTGAACACAACC

nucleotide sequences data were as followed.

Phylogenetic tree showed that HBoV strain detected by VP1/VP2 gene-based PCR and identified by sequencing and phylogenetic analysis have the most closest phylogenetic relationship with HBoV1 strains (Fig. 4).

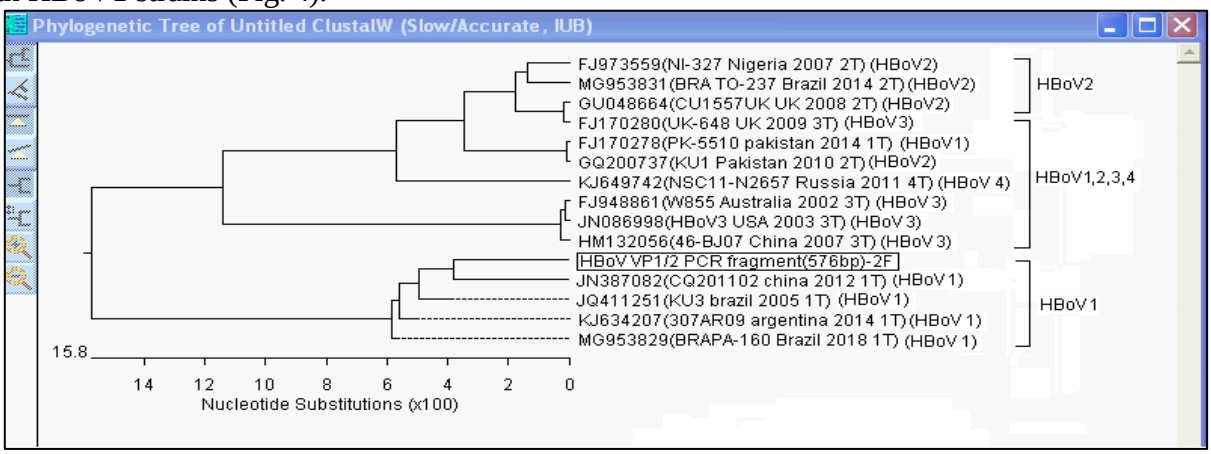

Fig. 4. Phylogenetic tree of the VP1/VP2 coding regions of HBoV strains constructed by the clustal method.

In addition to, HBoV strain detected and identified on basis of VP1/VP2 gene region showed very high sequence homology of 96.2-96.8% with different HBoV1 genotypes, and phylogenetic analysis also showed that this virus belonged to the HBoV1 genotype.

3.2.2. Identification of HBoV based on NS1 gene specific amplication bands

The products (0.35kb) from positive HBoV NS1 gene specific PCR were purified and sequenced in reverse primer dierrections. As result, 294 nucleotide sequences were analyzied from the amplified products (0.35kb) of HBoV NS1 gene. The analyzied nucleotide sequences data were as followed.

GTTCTGGAAGCGGCAGCCCGTTAATAAGAGTGTGAGAGTAAGTTTTTTCAGCTAAAATGAACCAGTTGTCAGGAGAAGTACAAACATCA
GGTTTGCTGTAAGATGAAATACATCTATTTTTAGGTAAAAGGTAGTTTTTGAAGAAGCGAAGAGGATCCACTGTTTGTGCATGAAGGTCTCCT
CTGCGATCTCTATATTGAAGGATCTGCATGTTGCCGCCAGTAACTCCACCCCATGCCTCTCGCTCAGCCTTTTTTAGAGTGTGAAATATGTCAG
CCTCCTCAGGTTCATAGG

Phylogenetic tree showed that HBoV strain detected by NS1 gene-based PCR and identified by sequencing and phylogenetic analysis have the most closest phylogenetic relationship with HBoV1 strains (Fig. 5 and 6).

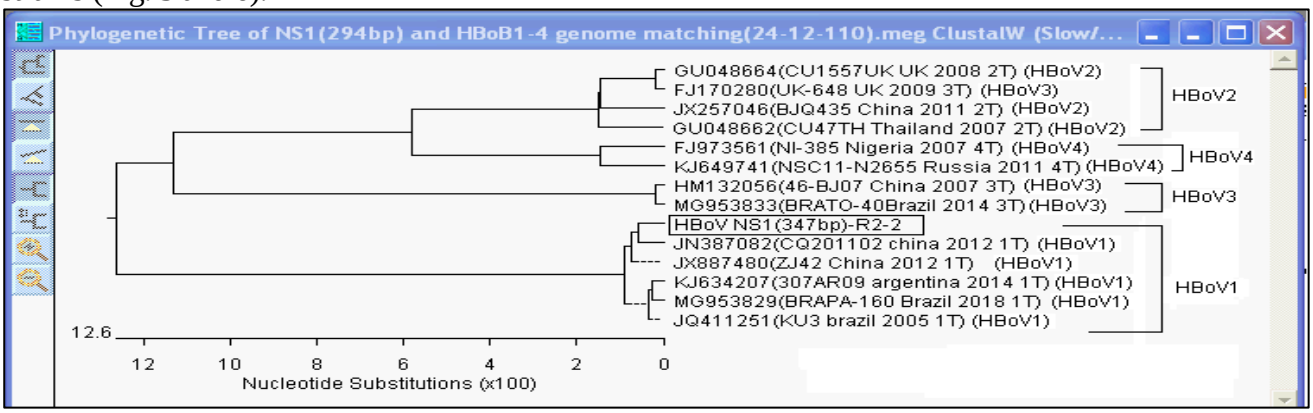

Fig. 5. Phylogenetic tree of the NS1 coding regions of HBoV strains constructed by the clustal method.

In addition to, HBoV strain detected and identified on basis of NS1 gene region showed very high sequence homology of 98.3~99.9% with different HBoV1 genotypes, and phylogenetic analysis also showed that this virus belonged to the HBoV1 genotype.

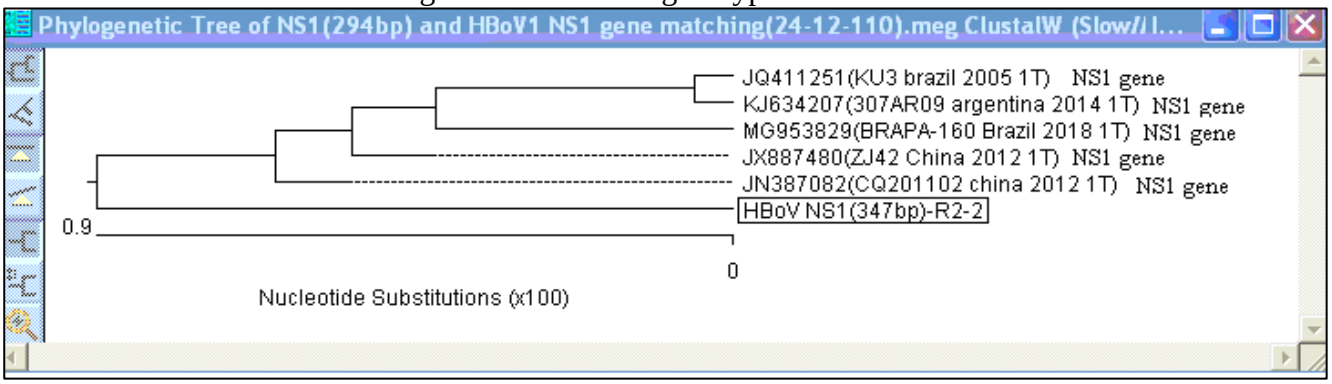

Fig. 6. Phylogenetic tree of the NS1 coding regions of HBoV 1 genotype strains constructed by the clustal method.

3.3. co-infection with other viruses

To determine the co-infection with other viruses, we conducted RT-PCR detections to 3 nucleic acid extracts positive in HBoV VP1/VP2 and NS1 genes-based PCR using primers specific for influenza viruses A (H1N1) and A (H3N2), human respiratory syncytial virus (HRSV), human parainfluenza virus (HPIV)1 and 3 types, and human metapneumovirus (HMPV) (Fig. 7).

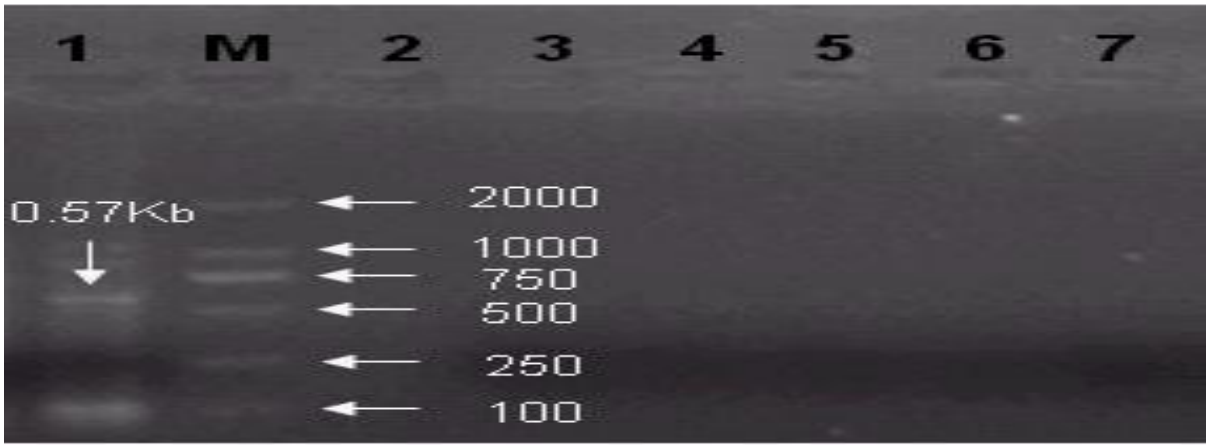

Fig. 7. RT-PCR for detection of co-infection with other viruses : lane 1: PCR product specific to HBoV VP1/VP2 gene, lane 2: RT-PCR product specific to HMPV, lane 3: RT-PCR product specific to influenza virus A(H1N1), lane 4: RT-PCR product specific to influenza virus A(H3N2), lane 5: RT-PCR product specific to HRSV, lane 6: RT-PCR product specific to HPIV 1, lane 7: RT-PCR product specific to HPIV 3, lane M: DL2000 molecular marker (1.5% AGE)

As a result, only HBoV1-specific amplicons were visualized in the HBoV positive NPA specimens, whereas influenza virus A (H1N1) and A (H3N2) types, HRSV, HPIV 1 and 3 types, and HMPV were not detected.

3.4. Clinical findings and outcomes of patients infected with HBoV1

Clinical presentation and outcomes of the children infected with HBoV1 newly discovered in 2005 were very interesting (Tab 1). HBoV1 was detected in 50% (3/6) of the patients hospitalized with asthma/wheezing. All Children infected with HBoV1 are male in gender and ranged from 11 month to 20 months of age (median 16.7 months). All Children infected with only HBoV1 had mild–to–moderate fevers (37.8~38.5℃). Leukocyte counts were normal or moderately elevated and oxygen saturation were fellen remarkedly. Chest radiographs obtained for 3 children with HBoV1 infections showed abnormalities such as hyperinflation and interstitial infiltrates. The most common and main clinical symptoms and signs of all HBoV1 infected patients at admission were cough, dyspnea, crackling rales, wheezing, cyanosis, stroke of asthma, hyperpnea, nausea, vomiting and other signs of respiratory distress.

The main clinical diagnoses of the HBoV1 infection-positive children were wheezy bronchitis (1 cases), acute paroxymal bronchiolitis (1 cases), and acute asthmatic bronchitis (1 cases). All 3 children (100%) with HBoV1 infection were prematurely born as premature infants between 32 and 36 weeks of gestation, with a lower birth weight and suffered from various degree of chronic respiratory diseases, which suggests that these children have an increased susceptibility to HBoV1 associated diseases such as acute asthma and wheezing. Oxygen saturation of the all patients was monitored during the hospital stay. Oxygen therapy was provided to achieve oxygen saturation ≥95%. The bronchodilator use and administration of antibiotics for each patient according to physical findings and laboratory data. were judged. All Children with HBoV1 infection recovered and were discharged within 4 to 9 days.

Overall, HBoV1 was the only virus detected in all 3 cases with acute asthma and wheezing. High HBoV1 copy number infection was preferentially seen in the absence of other viral agents, supporting its causative role for acute asthma and wheezing. These findings demonstrated an very close association between HBoV1 infection and occurrances of the acute respiratory infections such as asthma and wheezing. The result observed in our study were similar to that reported by previous studies (2). HBoV1 was the only infectious virus identified in 3 children with HBoV1 infection, which strongly suggests that it was the causative agent of the acute respiratory disease such as acute asthma and wheezing attacks.

Table 1. Clinical characteristics of children infected with HBoV1 at admission

Age (mo)	Sex	Fever (°C)	Leukocytes ($\times 10^3/\mu L$)	Saturation of oxygen (%)	Main Symptoms	Major Diagnosis	Underlying condition (wks of pregnancy)	Chest X ray Imaging	Severity of disease	Days in hospital
20	M	37.8	9.2	NA	C, D, W, RD, H, CR, HP	wheezy bronchitis	preterm(34)	hyperinflation	moderate	4
11	M	38.5	10.8	91	C, RD, W, H, FB, HP, SA, N, V	acute paroxymal bronchiolitis	Preterm(32) Chronic respiratory disease	interstitial infiltrates	severe	8
19	M	38.0	10.4	88	C, D, W, H, FB, SA, N, V	acute asthmatic bronchitis	preterm(36)	hyperinflation	moderate	5

Abbreviation [M: Male, C: Cough, D: Dyspnea, W: Wheezing, CR: Crackling Rales, RD: Respiratory Distress, H: Hypoxia, FB: Fast breathing, OA: Obstruction of the Airways, CN: Cyanosis, SA: Stroke of Asthma, HP: Hyperpnea, N: Nausea, V: Vomiting, NA: not available, B: Bronchiolitis, A: Asthma, HI: hyperinflation, II: interstitial infiltrates]

CONCLUSION

In this study, we detected human bocavirus genotype 1 (HBoV1) in three children hospitalized with acute asthma/wheezing by PCR using specific primers targeting the VP1/VP2 and NS1 genes and confirmed that the virus detected by HBoV1 specific PCR was HBoV1 through DNA sequence analysis and Phylogenetic analysis of specific amplicons. In these children, HBoV1 is the only causative virus that causes symptoms and signs of acute respiratory diseases such as cough, dyspnea, asthma, wheezing, hypoxia, cyanosis and respiratory distress, suggesting that sole HBoV1 infection is one of the decisive causes of acute asthma and wheezing attacks in children.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Author Contribution

Chang Guk Ri: Overall design, primer design, PCR detection, Hyok Kim and Yong Chan Sin: Analysis of clinical characteristics, Yong Chol Pak: DNA sequence analysis and Phylogenetic analysis, Yong Su Song: PCR detection, Sin Hyok Paek: Analysis of Chest X ray Imaging and Laboratory data, Ryong Sik Kim: Collection of samples and statistical analysis.

Abbreviation: HBoV - Human Bocavirus , PCR - Polymerase Chain Reaction , DNA - deoxyribonucleic acid, NPA - Nasopharyngeal aspirate , NS - nonstructural protein , NP - nuclear phosphoprotein , VP1/2 - structural capsid protein 1 and 2 ,NCBI - National Center of BioInformation , URTI - upper respiratory tract infections , LRTI - lower respiratory tract infections ,HRSV - human respiratory syncytial virus , HPIV - human parainfluenza virus , HMPV - human metapneumovirus.

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