Human bocavirus in Jordan: prevalence and clinical symptoms in hospitalised paediatric patients and molecular virus characterisation

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ABSTRACT

Introduction: This study was aimed at investigating the prevalence of human bocavirus (HBoV) among Jordanian children hospitalised with lower respiratory tract infection (LRTI) as well as the clinical feature associated with HBoV infection, the seasonal distribution of HBoV and the DNA sequencing of HBoV positive samples.

Methods: A total of 220 nasopharyngeal aspirates were collected from children below 13 years of age who were hospitalised with LRTI in order to detect the presence of HBoV using real-time polymerase chain reaction assay and direct HBoV sequencing.

Results: HBoV was detected in 20 (9.1 percent) patients, whose median age was four (range 0.8–12) months. Children under the age of 12 months were more susceptible to HBoV infection (p-value is 0.016). The main clinical diagnoses of patients infected with HBoV were bronchopneumonia (35 percent) and bronchiolitis (30 percent). Coughing (100 percent), wheezing (82.7 percent) and fever (68.2 percent) were the most prominent symptoms in infected patients. HBoV infections were seasonal; increasing in cooler months, diminishing in the summer and peaking in March (45 percent). Direct DNA sequencing revealed that three out of 20 (15 percent) specimens were identical to Stockholm 1 and 2 isolates, and single base pair substitution (A to T) at codon 92 was found in 17 out of the 20 (85 percent) specimens that were positive for HBoV, resulting in a threonine-to-serine substitution.

Conclusion: More attention should be given to diagnosing HBoV in patients with LRTI using molecular techniques.

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INTRODUCTION

Despite the fact that human knowledge of the viruses that infect humans is still incomplete, viral infections lay a heavy disease burden on mankind.[1] Unidentified human viruses may exist, causing acute and chronic diseases. Recent studies have indicated that acute respiratory tract infection (ARTI) is a major factor responsible for the hospitalisation of infants and young children, and that it is also a major cause of morbidity and mortality worldwide.[2] Respiratory tract infection (RTI) is the third most common cause of death in children.[3]

Recently, human bocavirus (HBoV) has been identified as a new virus in the respiratory secretions of Swedish children with lower respiratory tract infections (LRTI).[1] It was given the name human 'bocavirus' because of its link to the genome organisation of two other parvoviruses, bovine parvovirus 1 and minute virus of canines.[4-6] This new virus is a member of the *Parvoviriadae* family, *Parvovirinae* subfamily and the genus of bocavirus.[7,8] Several studies have isolated HBoV from children with RTI worldwide, and the incidence was found to be 1.5%–19%.[9-15] However, the rate of co-infection with other high-rate respiratory viruses has made it difficult to draw conclusions about its pathogenicity.[16-20]

Few studies have focused on the presence of HBoV in individuals without respiratory symptoms,[5] thus suggesting the possibility that HBoV may also be present in healthy, asymptomatic individuals. In recent studies, HBoV has been identified in the blood[18] and stool samples of children with gastroenteritis.[21-23] Clinical observations of patients infected with HBoV have led to the primary assumption that fever, cough, pulmonary symptoms, rhinitis, bronchiolitis and severe pneumonia are the main symptoms of bocavirus infection.[6,11] This study was aimed at investigating HBoV infection in terms

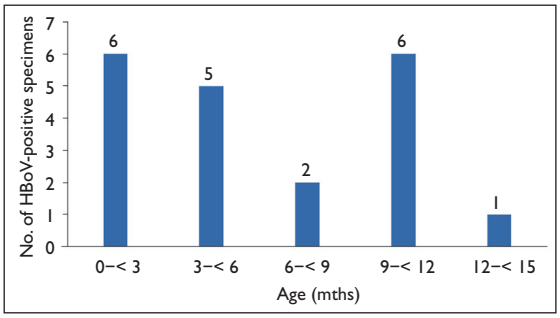

Fig. 1 Bar graph shows the aetiological agent in HBoV-positive children of different age groups.

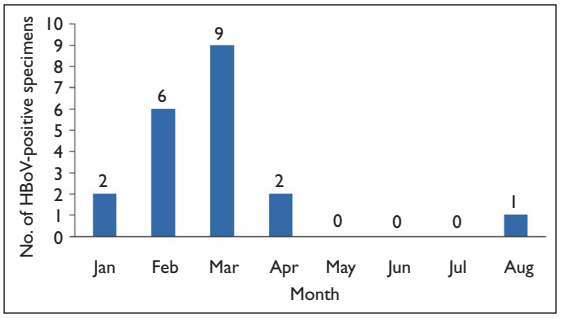

Fig. 2 Bar graph shows the monthly distribution of specimens that tested positive for HBoV.

of its prevalence among Jordanian children who were hospitalised with LRTI, its clinical features, seasonal distribution and the DNA sequencing of HBoV-positive samples.

METHODS

A total of 220 nasopharyngeal aspirate (NPA) specimens were collected from children aged > 13 years who were hospitalised with LRTI at the Princess Rahma Hospital in North Jordan from January to December 2007. Patients with congenital and persistent respiratory diseases were not included in this study. Each sample was processed on the same day of collection. When immediate processing was not possible, the sample was stored at –70˚C pending processing. Specimens were tested for the presence of HBoV by real-time polymerase chain reaction (PCR).

Viral nucleic acids were extracted from the patients' NPA using the High Pure Viral Nucleic Acid Kit (Roche, Mannheim, Germany), according to the protocol described by the manufacturer. The nucleic acid was eluted in a 50 µl buffer and stored at –70˚C until the time of analysis. The PCR product for a positive NPA specimen was cloned into pJET1.2/blunt cloning vector (Fermentas, Leon-Rot, Germany). Plasmids were purified using the QIAGEN Plasmid Mini Kit (Qiagen, Hilden, Germany) and sequenced in forward and reverse directions using a BigDye® Terminator Cycle Sequencing Kit version 3.1 (Qiagen, Hilden, Germany) and the ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA) for verification (GenBank accession ID DQ000496 and ID DQ000495) and used as a positive control in both conventional and real-time PCR run.

Real-time PCR reactions were carried out in a total volume of 25 µl which consisted of 12.5 µl IQ Supermix (Bio-Rad, Hercules, CA, USA), 2 µl (0.5 µM) NP-1 forward primer (5'-AGA GGC TCG GGC TCA TAT CA-3'), 2 µl (0.5 µM) NP-1 reverse primer (5'-CAC TTG GTC TGA GGT CTT CGAA-3'), 0.4 µl (0.1 µM) NP-1 probe (5'-6-FAM-AGG AAC ACC CAA TCA RCC ACC TAT CGT CT 3'-BHQ1) 5 µl of nucleic acids and 8.1 µl of nuclease-free water. Amplification was carried out on a MX4000™ Stragene real-time PCR machine (Stragene, La Jolla, CA, USA). Parameters of amplification were implemented according to the protocol of Lu et al;[24] 3 min at 95ºC for activation of iTaq DNA polymerase, and 45 cycles for 15 sec at 95ºC and 1 min at 60ºC. Each run included one positive template control and one no-template control (NTC).

PCR reactions were carried out for all positive specimens of HBoV detected by real-time PCR using one pair of primers to amplify the NP-1 gene, BoV188F (5'GAGCTCTGTAAGTACTATTAC-3') and BoV542R (5'-CTCTG TGTTG ACTGA ATACAG-3'), as described by Allander et al.[18] PCR reaction was carried out in a total volume of 25 µl and contained 12.5 µl master mix (Promega, Madison, WI, USA), 5.5 µl of nuclease-free water, 1 µl (200 pmol/ µl) of each primer and 5 µl of extracted DNA. The amplification protocol was as follows: initial denaturation for 10 min at 94ºC, followed by 35 cycles for denaturation at 94ºC for 1 min, annealing at 54ºC for 1 min, and extension at 72ºC for 2 min. The final extension step took 1 min at 72ºC. The amplification products were visualised on 2% gel electrophoresis to check the size of the amplified NP-1 gene. To avoid contamination, each run included a negative control with all PCR mix components except the DNA and the positive control.

Amplicons were purified using the EZ-10 Spain Column PCR Products Purification Kit (Bio Basic Inc, Markham, ON, Canada), and nucleotide sequencing reactions were performed on forward direction using the BigDye Terminator Cycle Sequencing Kit. Cleaning of sequencing PCR products was done using either the DyeEx 2.0 spin purification kit (Qiagen, Hilden, Germany) or Centri-Sep Column Cleaning Kit (Promega, Madison, WI, USA). Sequences were determined using the ABI 310 DNA sequencer. The sequences of samples were analysed using ChromasPro Software version 1.34

Table I. Clinical diagnosis and signs and symptoms in 20 HBoV-positive patients.

Parameter	No. (%) of HBoV-positive patients
Clinical diagnosis	
Bronchopneumonia	7 (35)
Bronchiolitis	6 (30)
Bronchiolitis and wheezing chest	1 (5)
Wheezing chest	1 (5)
Pneumonia	1 (5)
Other respiratory infection	4 (20)
Signs and symptoms	
Cough	20 (100)
Wheezing	18 (90)
Fever	12 (60)
Tachypnoea	10 (50)
Retraction	10 (50)
Crepitating	6 (30)
Vomiting	2 (10)
Cyanosis	1 (5)
Grunting	1 (5)
Diarrhoea	1 (5)
Conjunctivitis	0 (0)
Other	2 (10)

(available at: www.technelysium.com.au/ChromasPro.html). Blast N (available at: blast.ncbi.nlm.nih.gov/Blast.cgi) was used for comparison of the obtained sequences to nucleotide sequence databases, and for alignment of the obtained sequence to the nucleotide sequence of reference (ID DQ000495 and ID DQ000496). Data were collected and analysed using the Statistical Package for the Social Sciences version 15.0 (SPSS Inc, Chicago, IL, USA). Fisher's exact test was used to test the correlation between discrete variables. Alpha was taken as 0.05 in all analyses.

RESULTS

From January to December, 2007, a total of 220 NPA specimens were collected from children below 13 years of age who were hospitalised with lower respiratory illnesses. The median age of the children was six (range 0.5–132.0) months. The male to female ratio was 1.78:1. Human bocavirus was detected in 20 (9.1%) specimens. The male to female ratio of HBoV-positive children was 1.5:1, and their median age was four (range 0.8–12.0) months. The age distribution of the HBoV-positive patients is shown in Fig. 1. HBoV was detected in 19 (95%) children below one year of age. There were six (30%) patients in each group of patients aged 0 to < 3 months and 9 to < 12 months. HBoV infection was strongly associated with patients < 1 year of age ($p = 0.016$). Co-infection with respiratory syncytial virus (RSV) was detected in four (20%) HBoV-positive specimens (data not shown).

The clinical diagnosis and frequency of signs and symptoms among children with HBoV are shown in Table I. The main clinical diagnoses in HBoV-positive patients were bronchopneumonia (35%) and bronchiolitis (30%). Cough (100%), wheezing (90%) and fever (60%) were the most prominent symptoms in infected patients. The mean hospitalisation period for the 20 HBoV-positive children was 4.1 days and the median was four (range 1–13) days (data not shown). 17 (85%) patients who were positive for HBoV had taken antibiotics (data not shown). The monthly distribution of HBoV infection is shown in Fig. 2. Most infections with HBoV were reported from January to April 2007. The peak incidence of HBoV was in March (n = 9, 45%), followed by February (n = 6, 30%). Only one case was reported in August.

Direct sequencing was performed for all HBoV-positive specimens in forward direction. Compared with the reference isolates ST1 and ST2 (GenBank DQ000495 and DQ000496), an identical sequence was obtained in three isolates. One variation (A–T) was identified in each of the 17 specimens predicting the amino acid change from T (ACT) to S (TCT) in codon 92.

DISCUSSION

HBoV was first isolated from 17 (3.1%) Swedish children with LRTI;[1] thereafter, several studies have demonstrated that HBoV is prevalent in children suffering from URTI and LRTI, with the infection being more associated with LRTI.[4,9] We found HBoV in 20 out of 220 (9.1%) hospitalised children with LRTI over a one-year study period. Worldwide variations in the prevalence of HBoV are reported to be 1.5%–19%.[1,9,25,26] In Jordan, the prevalence of HBoV in our study was lower than that reported by Kaplan et al (57/312, 18.3%).[10] This may be due to the use of more specific assay, real-time PCR for the diagnosis of HBoV compared to the conventional PCR used by Kaplan et al.[10] Furthermore, in Kaplan et al's study, collection of NPA specimens commenced in December 2003 and from January to May 2004; during this period, there might have been a break in the spread of the HBoV.[10] A high incidence of HBoV co-infection in children with RTI has been reported in previous studies,[10,16,18] whereas the co-infection rate with RSV was 20% in our study (data not shown).

Seasonal occurrence of HBoV has been reported in different countries. In Sweden and France, HBoV was detected in the winter and spring months,[1,27] while it was more frequently detected from September to February in Hong Kong,[28] and in winter and autumn in Australia.[2] No apparent seasonal prevalence was observed in Canada and Germany.[9,11] Our study

demonstrated that HBoV infections in Jordan are mostly detected during the respiratory illness season, from January to April, and peak in March (45%). Similar findings have been reported in many other countries.[1-2,4,5]

The most frequent clinical syndromes associated with HBoV infection include pneumonia, bronchiolitis and wheezing chest.[19,29] In the present study, bronchopneumonia and bronchiolitis were the major clinical syndromes associated with HBoV-positive patients. Cough, rhinorrhoea and fever were the most frequent clinical signs and symptoms associated with HBoV infection.[28,29] In our study, the two major clinical signs and symptoms noted were cough (100%) and wheezing (90%). Oral or intravenous antibiotics were given excessively (94.1%) to infected patients, of whom 85% were positive for HBoV (data not shown). The high percentage of antibiotic therapy among our patients may be attributed to the unavailability of routine laboratory diagnosis of viral illness in Jordan; therefore, paediatricians regularly prescribe antibiotics to their patients to avoid secondary bacterial infections.[30]

The NP-1 gene is a highly conserved region in HBoV.[13,31] However, variations in the HBoV NP-1 region were demonstrated in several studies.[10,32] Direct sequencing of the NP-1 region recorded that three out of 20 amplicons had the same sequence as the original Swedish isolates ST1 and ST2. We found 17 amplicons with a single base pair substitution (A–T) that occurred in codon 92, resulting in an amino acid change from threonine to serine. This variation was reported for the first time in Jordan. Sequencing of the NP-1 gene uncovered a homology of more than 99% between our specimens. Also revealed was a homology of 100% between our specimens and the sequence of the NP-1 gene recorded in GenBank. We observed a homology of more than 98% between the amino acid sequence of our specimens and that of the Swedish isolates ST1 and ST2.

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