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Original Article

Detection of Human Bocavirus in Children with Acute Respiratory Tract Infections in Lanzhou and Nanjing, China

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Abstract

Objective The aim of this study was to explore the prevalent characteristics of HBoV1 and its co-infection.

Methods PCR was used to detect HBoV1-DNA (HBoV1) and other viruses. A multivariate logistic regression model was used to explore possibility of co-detected for related viruses.

Results The positivity rates in Nanjing and Lanzhou were 9.38% (74/789) and 11.62% (161/1386), respectively (P>0.05). The HBoV1 positive group was younger than negative group (P<0.05). Seasonal differences were noted, with a higher frequency of infection in December and July. HBoV1-positive children [72.34% (169/235)] were co-infected with other respiratory viruses. Multifactorial analysis showed no correlations between HBoV1 and the clinical classification, region, gender, age, or treatment as an outpatient or in a hospital. Correlations were identified between HBoV1 infections with ADV (OR=1.53, 95% CI 1.03-2.28), RSV (OR=0.71, 95% CI 0.52-0.98), and IFVA (OR=1.77, 95% CI 1.00-3.13).

Conclusions Presence of HBoV1 in nasopharyngeal aspirates did not correlate with region or gender, although the prevalence of HBoV1 was higher in younger children. There were no correlations between HBoV1 and other variables, except for the season and ADV, RSV, or IFVA infections.

Key words: Human bocavirus; Child; Respiratory tract infections

INTRODUCTION

Respiratory infections are often the result of combined bacterial and viral infections1, and severe acute respiratory infections are the leading cause of mortality among children WORLDWIDE2. The traditional viruses known to cause respiratory tract infections include adenovirus (ADV), influenza virus (IFV) A and B, human rhinoviruses (HRV), and respiratory syncytial
New viruses have been discovered in recent years, including human metapneumovirus (hMPV)\textsuperscript{[3]}, the coronaviruses HCoV-NL63\textsuperscript{[5-6]} and HCoV-HKU1\textsuperscript{[5-6]}, as well as human bocavirus (HBoV) which was the first virus identified by molecular virus screening in 2005\textsuperscript{[7]}.

More than two-thirds of childhood pneumonia cases are associated with viral infections\textsuperscript{[8-9]}. Reports of HBoV in respiratory tract infections have increased rapidly in numerous countries and regions throughout the world\textsuperscript{[10-17]}, with HBoVs detected in 1.5%-19% of respiratory tract secretions and 0.8%-9.1% of fecal samples\textsuperscript{[12-13]}. Thus, increasing evidence suggests that HBoV1 is an important cause of respiratory tract infections\textsuperscript{[10-12,14]}. HBoV2\textsuperscript{[18]}, HBoV3\textsuperscript{[19]}, and HBoV4\textsuperscript{[20]} were isolated recently in stool samples from patients with diarrhea. All four HBoV genotypes can be detected in stool of patients with acute gastroenteritis, while HBoV1 and HBoV2 were reported in respiratory tract samples\textsuperscript{[12]}. However, pathogen testing of respiratory infected patient sputum found the presence of other viruses and bacteria in addition to HBoV1. Thus, it remains to be clarified whether high-rates of HBoV co-infection in the respiratory tract lead to disease, or represents only a concomitant viral infection. There is also a lack of large-scale HBoV studies determining whether there are ethnic and regional differences in HBoV detection rates, since the incidence and clinical presentation of HBoV vary widely. Furthermore, the epidemiological characteristics of HBoV1 and its relationships with other viruses need to be elucidated.

**MATERIALS AND METHODS**

**Patients and Samples**

From November 2007 to July 2011, nasopharyngeal aspirates (NPAs) were obtained from 2175 children <173 months of age with acute respiratory tract infection. All cases of children with acute respiratory tract infections were included in our study, data were collected using a standardized questionnaire, diagnostic criteria based on Practical Pediatrics (Seventh Edition), including: acute upper respiratory tract infections (including acute laryngitis, herpes angina, acute tonsillitis), acute bronchitis (including asthmatic bronchitis), pneumonia (including bronchiolitis), and acute exacerbation of bronchial asthma. Inclusion criteria: (1) patients with the ‘acute respiratory infection’ diagnostic criteria characteristics from inpatient or outpatient, (2) patients or legal guardian signed an informed consent form, (3) age of patients were <15 years, (4) the course of disease of patients were within 48 h. Exclusion criteria: (1) the patients did not meet the diagnostic criteria, (2) Although within 48 h, the patients had received anti-viral or anti-inflammatory drug treatment, (3) patients associated with cardiovascular, liver, kidney, hematopoietic system, or other serious diseases, (4) the sample of patients could not be completely collected. Of these samples, 1386 were from the First Hospital of Lanzhou University (Lanzhou, China) and 789 were from Nanjing Children’s Hospital, Medical School of Nanjing University (Nanjing, China). Acute respiratory tract infections were classified according to WHO definitions\textsuperscript{[21]}; children with symptoms of acute upper respiratory tract infection (URTI) and acute lower respiratory tract infection (LRTI) were diagnosed in the presence of characteristic manifestations of rhinitis, pharyngitis, tracheitis, bronchitis, pneumonia, and severe pneumonia. The clinical diagnostic classification (I-IV) was based on previous studies\textsuperscript{[22]}. It was assessed at the time of enrollment and categorized as follows. I grade: very mild (upper respiratory tract symptoms/signs only); II grade: Mild (lower respiratory tract symptoms/signs +/2 upper respiratory tract symptoms/signs but not needing hospital admission); III grade: moderate (lower respiratory tract symptoms/signs +/2 upper respiratory tract symptoms/signs, needing hospital admission but with oxygen saturations in air >93% on pulse oximetry); IV grade: severe (lower respiratory tract symptoms/signs +/2 upper respiratory tract symptoms/signs, needing hospital admission and oxygen with saturations in air <93%). Informed consent was obtained from the parents of all children who provided specimens and the study protocol was approved by the Ethics Committee of each hospital. Demographic and clinical data were recorded from all patients and specimens were stored at -80 °C for further analysis. Viral RNA and DNA were extracted from the NPAs by using a QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany), which extracts viral RNA and DNA simultaneously according to the manufacturer’s instructions.

**Detection of HBoV1 Using Nested PCR**

The nested PCR assay with primers (forward1 \textsuperscript{5'}-A GGTAACCAATATTGCAAAGGCCCAT AGTC-3')
and reverse1 5’-TGGGAGTTCTCTCCGTCCGTAT C-3’, forward2 5’-AGGGTTTG T CTTTAACGATTGC AGAC AAC-3’, and reverse2 5’-TATACACAGAGT CGTCAGCAC TATGAG-3’) was performed using Ex-Taq DNA polymerase (TaKaRa, Beijing, China) to amplify a 455-bp fragment targeting a portion of the NS1 genes, as reported previously [23]. The amplicons from a second-round of PCR were analyzed by 1.5% agarose gel electrophoresis and sequenced with the Big-Dye Terminator Cycle sequencing kit and the automated ABI Prism 310 Genetic Analyzer (Qiagen, Beijing, China). Sequences were edited using the DNA Star software package. BLAST (www.ncbi.nlm.nih.gov) was used to confirm gene identities in the National Center for Biotechnology Information (NCBI) databases.

Detection of Other Respiratory Viruses in Samples

All samples were tested for 11 pathogens using standard reverse transcription PCR. These pathogens included IFVA, IFVB, HMPV, RSV, HRV, PIV types 1-3, HCoV-NL63, and HKU1[7,24-26]. A different PCR method was used to detect ADV[27].

Statistical Analysis

All sample data were done using EpiData Analysis. Categorical data and the median ages of children in different groups were compared using the Chi-square (χ²) test and Mann-Whitney test, respectively. Binary logistic regression analysis and independent variables were defined by region (Lanzhou=1, Nanjing=2), gender (Male=1, Female=2), age (months), season (1-12), disease classification (I-IV), and inpatient/outpatient (inpatient=1, outpatient=2). The 11 other respiratory viruses were also defined as independent variables (positive=1, negative=0). HBoV1 infection (positive=1, negative=0) was the dependent variable in multivariate analysis. Eigenvalues and condition indices were used to assess multi-colinearity in the regression models. All tests were two-tailed and P<0.05 was considered statistically significant. All analyses were performed using SPSS version 13.0.

RESULTS

Patient Characteristics

The 2175 children with acute respiratory infections in this study were aged 9 d to 173 months (The median age was 26 months). The majority of patients (74.11%) were aged >24 months. The ratio of males to females was 2.0:1.0 and that of inpatients to outpatients was 6.35:1. The case ratio of Lanzhou to Nanjing was 1.76:1.0. Respiratory tract infection disease classifications at grade III-IV accounted for 66.73% of cases.

Detection of HBoV1 in Children with Respiratory Tract Infections

HBoV1-DNA was detected by nested PCR in 235 specimens, an overall frequency of 10.80%. HBoV1 positive rates were 11.62% (161/1386) in Lanzhou and 9.38% (74/789) in Nanjing (χ²=2.61, P>0.05). The ratio of males to females with HBoV1-DNA was 1.83:1 but the HBoV1 positive rate did not differ based on gender or region (Table 1). The positivity rates were 6.97% (53/760) for the <6 months old group, 14.55% (112/770) for the 7-24 months old group, 12.30% (53/431) for the 25-60 months old group, 13.64% (9/66) for the 61-72 months old group, 5.41% (8/148) for the >72 months old group (Figure 1). HBoV1-DNA was detected in every month, but detection peaked in December (13.75%), followed by 13.16% in July, 12.70% in February, 12.50% in April, and 11.29% in May (Figure 2). The seasonal distribution of HBoV1-DNA differed significantly (χ²=19.97, P<0.05) in the positive and negative groups.

Co-infection with Common Respiratory Pathogens

A majority of the HBoV1-DNA positive children [72.34% (169/235)] were co-infected with other respiratory viruses. Co-infection with four viruses was detected in one sample (0.59%), three viruses in 9 samples (3.62%), two viruses in 43 samples (25.44%), and one virus in 116 samples (68.64%). RSV had the highest frequency of HBoV1 co-infection.
in 38.46% (65/169) of patients, followed by HRV in 30.77% (52/169), ADV in 20.71% (35/169), PIV3 in 17.75% (30/169), IFVA in 9.47% (16/169), HMPV in 6.51% (11/169), NL63 in 4.73% (8/169), IFVB in 4.14% (7/169), PIV1 in 2.96% (5/169), and HKU1 in 2.37% (4/169). Co-infection with PIV2 was not detected.

**Multifactorial Analysis of HBoV1**

When 17 variables were excluded, multifactorial logistic regression analysis showed no correlations between HBoV1 and the clinical diagnostic classification, region, gender, age, or treatment as an outpatient or in a hospital. Seasons however, correlated significantly with HBoV1 infection [odds ratio (OR)=1.04, 95% confidence interval (CI) 1.00-1.08; \( P = 0.05 \)]. Regression analysis also showed that HBoV1 was positively correlated with co-infection of ADV (OR=1.53, 95% CI 1.03-2.28; \( P = 0.03 \)), RSV (OR=0.71, 95% CI 0.52-0.98; \( P = 0.04 \)), and IFVA (OR=1.77, 95% CI 1.00-3.13; \( P = 0.05 \)) (Table 2).

![Figure 1. Age distribution of HBoV1-positive patients.](image1)

![Figure 2. Seasonal distribution of HBoV1 from November 2007 to July 2011 (overall values of those obtained in the respective months).](image2)
DISCUSSION

HBoVs have been detected in respiratory samples\textsuperscript{[28–29]}, stool samples\textsuperscript{[30–31]}, urine samples\textsuperscript{[32]}, and blood samples\textsuperscript{[33]} derived from children and adults worldwide. In patients with upper or lower respiratory disease, HBoV1 is predominantly a respiratory associated pathogen, whereas HBoV2, HBoV3, and HBoV4 have been found mainly in stool\textsuperscript{[12]}. HBoV1 was found in 2%-19% of patients\textsuperscript{[13]}. In children, the HBoV detection rate in throat swabs was low at 3%-11.8%\textsuperscript{[14, 28, 34]}, but detected at higher rates of 10.3%–22.5% in NPA samples\textsuperscript{[11, 35]}. Obvious gaps in the detection rate were observed in different studies and regions with respiratory infections\textsuperscript{[12–13]}. HBoV1 was rarely detected in adults compared to infants and young children\textsuperscript{[16–37]}. The HBoV1 positive rate was 0.3%-1% in 16-50 year-old population in Canada\textsuperscript{[25]}, 33% in the 0-2 year-old\textsuperscript{[29]}, and 17% in the <5-year-old patients\textsuperscript{[13]}. These multiple pieces of evidence strongly implied that HBoV1 is an important respiratory pathogen in children. Our data in this study indicated that the HBoV1 detection rate was higher in children 6-72 months of age but lower in children <6 months of age or >72 months of age, which is similar to previous studies\textsuperscript{[38–40]}. It is unknown whether the detection rate was impacted by immunization.

The epidemiological characteristics of HBoV1 demonstrated detection of HBoV1 in 10.8% of young children, representing 9.39% of patients from Nanjing and 11.62% of patients from Lanzhou. In our study, the distribution of detection rate difference in gender was not statistically significant between the two cities, a finding consistent with related studies\textsuperscript{[10–11, 14]}. Additionally, the detection rate of HBoV1 between the two cities in our study did not show statistical significance, a result which could suggest a difference in patient population surveyed, or the testing methods used to include and exclude patients involved in the study. Previous studies have reported the HBoV1 detection rate in children with acute respiratory infection to be 3% in Guangzhou\textsuperscript{[12, 14]}, 7%\textsuperscript{[41]}, and 10%\textsuperscript{[38]} in Shanghai, 10% in Germany\textsuperscript{[39]}, and 7% in New Delhi\textsuperscript{[42]}. In this study, the HBoV1 detection rate in Nanjing and Lanzhou was significantly higher than in Guangzhou. This may be explained by sampling differences and study population age, as pharyngeal swabs were used in a study of patients <18 years old in Guangzhou. This indicates that the sample source, disease classifications, population age structure and detection method might be critical to HBoV1 detection. The detection rate of HBoV1 mostly were higher in winter and spring than other seasons in different countries and regions\textsuperscript{[13, 37]}. We speculate that it may be related to higher incidence of respiratory infections during these seasons. However, seasonal differences of HBoV1 detection rates still need to be investigated further in the same area.

Table 2. Multivariate Analysis of HBoV1-DNA Positive Children Suffering from Respiratory Tract Disease

| Factor                          | $B$  | Wald | $P$  | OR   | 95.0% CI for OR |
|---------------------------------|------|------|------|------|-----------------|
|                                 |      |      |      |      | Lower           |
| Regions                         | -0.27| 2.74 | 0.10 | 0.76 | 0.55            |
| Gender                          | 0.09 | 0.35 | 0.55 | 1.09 | 0.82            |
| Age                             | -0.00| 2.29 | 0.13 | 1.00 | 0.99            |
| Season (1-12 months)            | 0.04 | 3.77 | 0.05 | 1.04 | 1.00            |
| Diagnostic classification (I-IV)| 0.13 | 2.00 | 0.16 | 1.14 | 0.95            |
| Inpatient/outpatient            | -0.20| 0.81 | 0.37 | 0.82 | 0.54            |
| Adenovirus                      | 0.43 | 4.50 | 0.03 | 1.53 | 1.03            |
| Respiratory syncytial virus     | -0.34| 4.32 | 0.04 | 0.72 | 0.52            |
| Human metapneumovirus           | -0.09| 0.07 | 0.80 | 0.92 | 0.48            |
| Influenza A virus               | 0.57 | 3.84 | 0.05 | 1.77 | 1.00            |
| Influenza B virus               | 0.63 | 2.18 | 0.14 | 1.88 | 0.81            |
| New coronavirus (HCoV-NL63)     | -0.06| 0.02 | 0.89 | 0.96 | 0.52            |
| Human coronavirus (HKU1)        | -0.06| 0.01 | 0.92 | 0.95 | 0.33            |
| Parainfluenza virus type 1      | 0.33 | 0.45 | 0.50 | 1.40 | 0.53            |
| Parainfluenza virus type 2      | -1.74| 0.00 | 1.00 | 0.00 | 0.00            |
| Parainfluenza virus type 3      | -0.02| 0.01 | 0.91 | 0.97 | 0.64            |
| Human rhinovirus                | 0.27 | 2.39 | 0.12 | 1.31 | 0.93            |
| Constant                        | -2.05|      |      |      |                 |
While our study used highly sensitive and low error probability nested PCR to screen HBoV1, HBoV1 detection rates vary widely. Since our techniques result in highly precise and sensitive detection of HBoV1 infection, environmental or socio-economic factors may potentiate different patient risk factors. Previous studies have shown that the peak of HBoV1 infection is in the summer or spring. In our study however, the peak seasons of HBoV1 infection occurred in February, April, July, and December during the 48 months collecting period. Thus, the seasonal frequency of HBoV1 infection should be further investigated as it appears to be more common in the colder seasons. Regardless, the seasonal peaks of HBoV1 infection have differed among counties and climate regions.

Previous studies have determined that HBoV1 has a high co-infection rate with other respiratory viruses, but its pathogenic role was still debatable due to high frequency of co-infection. HBoV1 may exist in the respiratory tracts as a bystander without causality to a patient’s current symptoms. Additionally, HBoV1 may have an important role in acute respiratory infections among children. In patients with respiratory complaints, HBoV1 can be found alone or, more often, in combination with other viruses known to cause respiratory complaints. HBoV1 may be a factitive with other respiratory viruses for the observed symptoms, suggested by the findings that asymptomatic children had higher HBoV1 viral load than asymptomatic children. In our study, 72.34% of HBoV1-positive patients had co-infection with 10 different respiratory viruses, which is similar to previous reports. HBoV1 co-infection has been reported with RSV, IFVA, and ADV in children and adults with respiratory tract infections, and also with HMPV infections in children with community-acquired pneumonia. Thus, the reasons underlying the high co-infection rate of HBoV1 need further study and leads to questions regarding HBoV1 pathogenicity. Recent studies based on epidemiological evidence show that HBoV1 was likely to be a major contributor to acute respiratory tract disease, particularly in infants and children. HBoV1 co-infections with other viruses have been associated with greater disease burden (i.e., more hospitalizations and loss of school days) in children with respiratory tract infection than in those with HBoV infection alone. Many factors might have an impact on HBoV1 infection and a larger study seemed to corroborate a synergistic relationship between HBoV1 and the pathogenesis of respiratory syndromes such as influenza-like illnesses, bronchiolitis, and pneumonia. However, our regression model showed that HBoV1-positivity was highly correlated with season, RSV, ADV, and IFVA (P<0.05).

HBoV1 belongs to the parvoviridae family with viral head-to-tail genome sequences that use typical rolling circle replication. HBoV1 may be triggered by helper viruses, although it had been hypothesized that picornaviruses may be a factor that promotes clinical illnesses. Our analysis suggested a possible synergistic effect between HBoV1 and ADV and IFVA (OR>1). The results should be confirmed by further clinical and experimental data. Nevertheless, given that the OR value for RSV infection in multivariate analysis was <1, it was estimated that related to the wide distribution of RSV. Unfortunately, the analysis results did not show a link between age and HBoV1, which may be due to the fact that infants and young children were the primary participants in our study. This connection requires further investigation before any interactions can be confirmed.

Deficiencies still existed in our manuscript because some patients or legal guardians did not agree to take part in our study. As such, some cases were missed, so potential sample selection bias were not absolutely avoided. However, our large sample size likely weakened any potential sample bias. Samples of cases that were excluded were not collected in this study, so the frequency of infection in exclusive samples of cases needs to be revealed by more studies. In addition, distribution of HBoV1 positive infections in children without respiratory tract infections remains crucial to be explored for revealing relationships between HBoV1 in the health and the diseased.

In summary, we examined HBoV1 in children with respiratory infections but require further information to elucidate its pathogenic role. Our understanding of this virus would be greatly enhanced by large-scale molecular epidemiological studies of pathogens in different regions.

COMPETING INTERESTS

None of the authors has a conflict of interest.

AUTHORS' CONTRIBUTIONS

ZD and YJ designed the study. CC, XY, JS, JZ, YZ,
and XG collected the samples. JW, JL, ZX, and XG performed the PCR tests. JW and NL analyzed the data. JW, JY, and NL wrote and finalized the manuscript. All authors read and approved the final manuscript.

Received: April 20, 2014;
Accepted: July 1, 2014

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