Correlation between nucleotide mutation and viral loads of human bocavirus 1 in hospitalized children with respiratory tract infection

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The human bocavirus 1 (HBoV1) parvovirus causes respiratory disease and primarily affects children. Despite its worldwide prevalence, the mechanisms of HBoV1 replication and pathogenesis remain largely undefined. In this study of 846 children hospitalized at the Children’s Hospital of Chongqing Medical University in China for respiratory tract infection between June 2009 and May 2011, HBoV1 was detected in 112 (13.2 %) by real-time quantitative PCR. The median age of HBoV1-positive patients was 10 months old. Forty-five (40.2 %) of the HBoV1 cases were monoinfections, and 67 (59.8 %) were viral co-infections. Genotyping of all 112 HBoV1-positive cases yielded 27 full HBoV1 sequences, as well as two NS1 gene sequences, 15 NP1 gene sequences and 10 VP1/VP2 gene sequences harbouring 24, 10 and 43 mutations, respectively. Statistical analysis revealed no relationship between genetic mutations and clinical manifestations of HBoV1-positive patients. However, the viral loads were significantly lower in samples with mutations G236A or A447G in NP1, or G1461A in VP1/VP2, than in samples with wild-type HBoV1. Future studies should investigate whether these mutations in the HBoV1 gene may represent useful markers of disease pathogenesis.

INTRODUCTION

Viruses, such as the human bocavirus 1 (HBoV1) parvovirus, are important pathogenic agents of respiratory tract infections (RTIs) in infants. Since its initial detection in human respiratory tract samples, HBoV1 has been characterized as a ssDNA virus with a genome of approximately 5 kb that shows high structural similarity to the other two members of the bocavirus genus, bovine parvovirus and minute virus of canines (MVC) (Allander et al., 2005). The genome contains three ORFs that encode four proteins, including two non-structural proteins (NS1 and NP1) and two capsid proteins (VP1 and VP2). While very few studies have assessed the potential functions of NS1 and NP1, a recent report suggested that they may contribute to viral replication (Chen et al., 2010). Studies of the VP1 and VP2 genome structures revealed a shared identity at the C termini, and a distinctive feature at the N terminus of VP1 in which a unique protein, VP1-u, is encoded. Detailed analysis of VP1-u identified a conserved phospholipase A2 (PLA2) motif and characterized it as essential for infection (Allander et al., 2005; Qu et al., 2008).

Studies of HBoV1 replication have largely relied on the pseudostratified human airway epithelium cell culture system, according to its morphological and functional resemblance to human airways in vivo. Using this system, Dijkman et al. (2009) showed that the HBoV1 transcription mechanism is very similar to that of the other bocaviruses. Another study by Lüsebrink et al. (2011) identified the head-to-tail structure of HBoV1, and characterized it as a replicative feature that distinguishes HBoV1 from the other parvoviruses, which are composed of head–head, tail–tail structures.

The GenBank/EMBL/DDBJ accession numbers for the full and partial sequences of human bocavirus 1 determined in this study are JX434034–JX434085 and JX445150–JX445151.
A recent clinical investigation detected HBoV1 in numerous samples from the respiratory tract, faeces and blood, and suggested its potential as an aetiologic cause of the patient’s symptoms, including cough, fever, rhinorrhea, hypoxia and wheezing (Jartti et al., 2012). However, a previous study had reported that HBoV1 is often present as a co-infection with other respiratory viruses and suggested that only high viral loads ($>10^4$ copies ml$^{-1}$) of HBoV1 are associated with respiratory symptoms (Allander et al., 2007). In support of this, subsequent studies of nasopharyngeal aspirates (NPAs) of HBoV1 patients demonstrated high viral loads in 91% of patients with enhanced IgG antibody or seroconversion (Kantola et al., 2008) and in 96% of wheezing children (Söderlund-Venermo et al., 2009).

PCR and antibody detection (IgM and IgG) remain the most reliable methods for diagnosing acute HBoV1 infection; recently, however, quantitative real-time PCR of NPAs has emerged as a promising diagnostic tool for many acute RTIs. To take advantage of this new technology and develop an effective methodology, the genomic features of HBoV1 must be more fully elucidated. Few studies to date have addressed the relationship between natural genetic variation in HBoV1 and clinical manifestation and none have investigated the potential correlation with viral loads. To this end, NPAs were collected from 846 children hospitalized with RTI at our institute and used to investigate the distribution of HBoV1, its genetic variants and correlation with viral loads.

**RESULTS**

**Patient characteristics**

For the 846 RTI cases in this study, the median age was 7 months old (range: 1 month old to 16 years old). The majority of cases were between 1 month and 1 year old ($n=534$, 63.1%) and the amount of cases decreased with increasing age (1–2 years-old: $n=136$, 16.1%; 2–3 years old: $n=63$, 7.4%; >3 years old: $n=113$, 13.4%). The overall male:female ratio was 566:280 for a 2.0 bias towards males. The majority of RTI cases involved the lower respiratory tract (729/846, 86.2%), for which the most frequent diagnosis was bronchial pneumonia (217/729, 29.8%). Underlying conditions, such as congenital cardiac and pulmonary conditions or primary immunodeficiency, were present in 10.4% of the total 846 patients.

**HBoV1-associated RTIs**

PCR detected more than one virus (co-infection) in 72.2% of the 846 children with RTI. HBoV1 was detected in 13.2% (112/846) of the patients, among which 45 (40.2%) cases were monoinfections and 67 (59.8%) were co-infections. The HBoV1 co-infections most frequently involved respiratory syncytial virus (RSV; 30.4%), followed by parainfluenza virus (PIV; 19.6%), influenza virus (IV; 9.8%), adenovirus (ADV; 9.8%), coronavirus (CoV; 2.7%) and human metapneumovirus (hMPV; 1.8%).

**Clinical characteristics of HBoV1-positive patients**

The median age of the HBoV1-positive patients was 10 months old (range: 1 month old to 14 years old). There were significantly more HBoV1-positive patients in the 6 months-old and 2 years-old age group than in the >2 years-old group ($60/327$ versus $52/519$, $P<0.001$), but the male:female ratio of HBoV1 cases was similar to that of the total study population ($72:40$, 1.8 bias towards males). The highest rate of HBoV1 infections was detected in the summer ($41$ HBoV1-positive/196 RTI versus all other months: $81/650$, $P=0.003$) (Fig. 1). The most common respiratory symptoms of the HBoV1-positive patients were cough (98.2%), sputum (78.6%), wheezing (56.2%), fever (37.5%) and diarrhoea (33.0%).

**HBoV1 sequence analysis**

Each RTI case was subjected to HBoV1 sequence analysis for the NS1 gene (29 full sequences published in GenBank, accession numbers JX434059–JX434085, JX445150–JX445151), the NP1 gene (32 full and 10 partial sequences),

![Fig. 1. Seasonal distribution of HBoV1 and the positive rate in each month among hospitalized children with RTI (June 2009–May 2011).](image-url)
and the VP1/VP2 gene (32 full and five partial sequences). When the sequencing results were compared with the HBoV1 isolate st2 reference strain (GenBank accession no. DQ000496), 17 (58.6 %) of the 29 NS1 sequences contained mutations; the nucleotides of the NS1 gene and amino acid sequences from our study cohort varied within the range of 0–0.2 and 0–0.3 %, respectively. In total, NS1 mutations were found at 24 positions, 11 of which led to amino acid change (Tables 1 and 2).

For the 32 NP1 sequences tested, 31 (96.9 %) showed mutations. The nucleotides of the NP1 gene and amino acid sequences from our study cohort varied in the range 0–0.6 and 0–0.9 %, respectively. In total, NP1 mutations were found at 10 positions, three of which led to amino acid change (Tables 1 and 3).

All 32 (100 %) of the VP1/VP2 sequences tested showed mutations. The nucleotides of the VP1/VP2 gene and amino acid sequences from our study cohort varied in the range 0.1–0.4 and 0–0.6 %, respectively. In total, VP1/VP2 mutations were found at 43 positions, seven of which led to amino acid change. In addition, six of these 43 mutations occurred within the VP1-u region, four of which led to amino acid change (Tables 1 and 4).

Seven mutation sites in 42 of the NP1 sequences and nine mutation sites in 37 of the VP1/VP2 sequences were detected in three or more samples of our study cohort; in contrast, none of the mutation sites in NS1 were detected in three or more samples. When each genetic mutation (representing natural genetic variation) was assessed in relation to clinical symptoms, no significant relationship was found (data not shown). However, samples with mutations at 236 or 447 nt of NP1 or 1461 nt of VP1/VP2 did correspond to patients with lower virus copy number. For the 42 NP1 sequences tested, seven had a G→A transition at the 236 nt position, and the viral loads corresponding to these seven samples were significantly lower than those of the other 35 (median: 1.3×10⁶ versus 4.7×10⁷ copies ml⁻¹, P=0.001) (Table 5). Nine sequences had an A→G mutation at the 447 nt position, and the viral loads corresponding to these nine samples were significantly lower than the other 33 (median: 4.4×10⁶ versus 6.5×10⁷ copies ml⁻¹, P=0.003) (Table 5). For the 37 VP1/VP2 sequences tested, 34 contained the 1461 nt mutation

Table 1. Nucleotide mutations in NS1, NP1 and VP1/VP2 genes of HBoV1

<table>
<thead>
<tr>
<th>Encoded protein*</th>
<th>Total*</th>
<th>Nucleotide substitution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Single sample</td>
</tr>
<tr>
<td>NS1</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>NP1</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>VP1/VP2</td>
<td>43</td>
<td>31</td>
</tr>
</tbody>
</table>

*29, 32 and 32 full sequences of NS1, NP1 and VP1/VP2 were compared with the HBoV1 st2 reference strain (GenBank accession no. DQ000496), and 24, 10 and 43 mutations were found, respectively.

Table 2. Amino acid variation of and the number of samples with HBoV1 NS1 mutation

<table>
<thead>
<tr>
<th>Location*</th>
<th>Mutation</th>
<th>No.†</th>
<th>Location*</th>
<th>Mutation</th>
<th>No.†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Synonymous</td>
<td>Non-synonymous</td>
<td></td>
<td>Synonymous</td>
<td>Non-synonymous</td>
</tr>
<tr>
<td>50</td>
<td>K→K</td>
<td>1</td>
<td>369</td>
<td>L→L</td>
<td>1</td>
</tr>
<tr>
<td>84</td>
<td>A→T</td>
<td>1</td>
<td>413</td>
<td>F→F</td>
<td>1</td>
</tr>
<tr>
<td>154</td>
<td>E→E</td>
<td>2</td>
<td>426</td>
<td>S→S</td>
<td>1</td>
</tr>
<tr>
<td>161</td>
<td>K→K</td>
<td>2</td>
<td>427</td>
<td>T→P</td>
<td>1</td>
</tr>
<tr>
<td>182</td>
<td>G→D</td>
<td>1</td>
<td>431</td>
<td>N→N</td>
<td>1</td>
</tr>
<tr>
<td>254</td>
<td>E→K</td>
<td>1</td>
<td>443</td>
<td>Y→S</td>
<td>1</td>
</tr>
<tr>
<td>262</td>
<td>A→G</td>
<td>1</td>
<td>449</td>
<td>L→L</td>
<td>1</td>
</tr>
<tr>
<td>263</td>
<td>T→I</td>
<td>1</td>
<td>485</td>
<td>G→G</td>
<td>1</td>
</tr>
<tr>
<td>283</td>
<td>W→G</td>
<td>1</td>
<td>493</td>
<td>K→K</td>
<td>1</td>
</tr>
<tr>
<td>321</td>
<td>E→E</td>
<td>2</td>
<td>567</td>
<td>D→N</td>
<td>1</td>
</tr>
<tr>
<td>347</td>
<td>G→G</td>
<td>1</td>
<td>625</td>
<td>D→N</td>
<td>1</td>
</tr>
</tbody>
</table>

*Amino acid position in the NS1 encoded protein.
†Number of samples in which the mutation was detected.
and the remaining three produced amplicons of insufficient length to detect this position. In the 34 sequences of sufficient length, nine had the G→A transition at 1461 nt, and the viral loads corresponding to these nine samples were lower than the other 25 (median: 1.2×10^7 versus 4.2×10^8 copies ml\(^{-1}\), \(P=0.032\)) (Table 5). For the three mutations of NP1 and VP1/VP2, only the 236 nt mutation led to an amino acid change (S→N at position 79), while the other two mutations were synonymous (Table 5).

### DISCUSSION

While HBoV1 infections have been reported in many countries across the globe, its association with RTI is what attracted the attentions of the healthcare and scientific communities. Now considered an important and common pathogen of paediatric RTI cases, researchers have begun to unravel the epidemiology of HBoV1 (Kaida et al., 2010; Körner et al., 2011). In our current clinical study, HBoV1...
DNA was detected in 112 (13.2%) of 846 hospitalized patients with RTI. Moreover, the study’s data amassed after 2 years of continuous surveillance in south-western China revealed that the detection rate of HBoV1 was the highest from June to August, suggesting a seasonal (summer) trend, which should be investigated further.

The crux of our current study was the genomic underpinnings of HBoV1 infection in paediatric RTI cases. As stated in the Introduction, the HBoV1 genome consists of two non-structural proteins (NS1 and NP1) and two capsid proteins (VP1 and VP2). The sequences encoding the VP1 protein also contain sequences for a unique and functionally critical protein (VP1-u) and overlap the VP2 coding region. Although the precise function of NS1 remains unknown, in vitro cell culture studies and genome comparative analyses suggest that it may be involved in transactivation of virus replication (Chen et al., 2010). In our study of HBoV1-positive samples, the NS1 gene was the most conserved region. Although 24 positions were found to be mutated in the NS1 genes detected, eleven of those led to amino acid change. However, only the amino acid variation from A154T of NS1 had been previously reported (Xiu et al., 2010), and the other ten non-synonymous mutations represent novel findings and merit further investigation to determine their functional roles in the disease pathogenesis.

The mid-ORF encoded NP1 has also been proposed as a potential regulator of virus replication (Chen et al., 2010). Of the 10 mutations in NP1 detected in our study’s HBoV1-positive samples, three led to non-synonymous mutations. Again, one (S79N) had been previously reported (Xiu et al., 2010).

The putative PLA₂ motif in VP1-u has been characterized as crucial for its enzymic activity (sPLA₂-like) (Qu et al., 2008), which is believed to mediate transfer of the viral genome from late endosomes/lysosomes in the host cell to the nucleus where viral replication occurs (Zádori et al., 2001). In contrast, the studies of the VP2 protein have so far revealed an extracellular role in the host, in which HBoV1 VP2 virus-like particles elicit the immune response (Lindner et al., 2008). In our HBoV1-positive samples, 43 mutations were detected in the VP1/VP2 sequence region. Of those, six were located in the VP1-u encoding sequence but not in the crucial motif that influences the sPLA₂-like activity (Qu et al., 2008). The VP1-u amino acid variations R17K and L40S, as well as the N474S of VP1/VP2, had been previously reported (Chieochansin et al., 2007).

A previous study of another member of the family Paroviridae, human B19 erythrovirus, reported a surprisingly high rate of evolutionary change over a relatively short period of time (Shackelton & Holmes, 2006). A similar rapid evolution rate was demonstrated for porcine parvovirus (Streck et al., 2011), which shows a similar genetic structural organization to HBoV1. In our study, the NS1 gene of HBoV1 was the most highly conserved among the samples studied, and the VP1/VP2 appeared to represent a hypervariable region. It is possible that HBoV1 also has a high rate of evolution, similar to the other paroviruses, but continuous detection of HBoV1 variation is necessary to investigate this hypothesis and determine its implications for management of the disease.

Among all the HBoV1 mutations detected in our study, three frequent nucleotide mutations have the most obvious potential to affect virus replication. Interaction between viruses and components of the host innate immune system is crucial for viral replication and survival. A previous molecular study of HBoV1 showed that NP1 can inhibit the production of an important host cytokine, beta interferon (IFN-β), by blocking association of the IFN regulatory factor 3 with the IFNB promoter (Zhang et al., 2012). This immune modulatory activity mediated by NP1 may be beneficial to HBoV1 infection. Moreover, another molecular study indicated that the HBoV1 NP1 can functionally replace the MVC NP1, partially restoring the replication of an NP1 knockout MVC infectious clone (Sun et al., 2009). In our study, samples of patients with lower viral load also carried HBoV1 with NP1 nucleotide mutations at 236 (G→A) or 447 (A→G). The NP1 G236A mutation leads to the S79N amino acid change. These results suggest that nucleotides 236 and 447 may represent important nucleotide positions in NP1 that could affect the virus replication. Since HBoV1 is difficult to cultivate in vitro and no appropriate animal model of HBoV1 infection has been established, the mechanisms of HBoV1 virus replication and related host immune response remains largely unknown.

No study to date has reported on the relationship of HBoV1 mutations and clinical manifestations of the disease in a paediatric RTI population. However, two previous clinical studies of parvovirus B19 did investigate such relationships with mutations in the virus’ VP region, but found no significant correlations (Erdman et al., 1996; Takahashi et al., 1999). Another study of B19 did provide some evidence to suggest that the 508 aa site in the NS

Table 5. Correlation of HBoV1 nucleotide mutation and viral loads

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mutation</th>
<th>Amino acid</th>
<th>Variability, n/total (%)</th>
<th>Mutation versus non-mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP1</td>
<td>G236A</td>
<td>S79N</td>
<td>7/42 (16.7)</td>
<td>1.3 × 10⁶ versus 4.7 × 10⁷</td>
</tr>
<tr>
<td>NP1</td>
<td>A447G</td>
<td>Synonymous</td>
<td>9/42 (21.4)</td>
<td>4.4 × 10⁶ versus 6.5 × 10⁷</td>
</tr>
<tr>
<td>VP1/VP2</td>
<td>G1461A</td>
<td>Synonymous</td>
<td>9/34 (26.5)</td>
<td>1.2 × 10⁷ versus 4.2 × 10⁸</td>
</tr>
</tbody>
</table>

http://vir.sgmjournals.org
protein may influence the clinical course and immune reactivity in patients with persistent B19 infections (Hemauer et al., 1996). Although we found no significant relationship between the HBoV1 mutations and clinical manifestations, we did observe a relationship between nucleotide change and viral load. Specifically, nucleotide mutations at position 236 and 447 of NP1 and at 1461 of VP1/VP2 were frequent in patients with significantly lower viral loads. In addition, other mutated nucleotides of HBoV1 that were frequent in our study cohort also occurred in patients with lower, but not significantly lower, viral loads (data not shown). In hepatitis B virus, two mutations in the core promoter (A1762T and G1764A) are known to moderately enhance viral genome replication (Parekh et al., 2003), and such a mechanism may be at play in HBoV1. Furthermore, Qu et al. (2008) reported that certain HBoV1 nucleotide mutations that affect amino acids in the VP1-u encoding region (i.e. 21, 41, 42 and 63 nt) nearly obliterate its sPLA₂-like activity (Qu et al., 2003), and such a mechanism may be at play in HBoV1. To determine whether the three most frequently detected mutations from our study (G236A and A447G of NP1; G1461A of VP1/VP2) influence HBoV1 replication or its pathogenesis, further investigation is required.

In conclusion, HBoV1 was detected in clinical samples from children with RTI and several mutations were detected throughout the viral genome. While none of these mutations showed a relationship to the patients' clinical manifestations, three mutations (G236A and A447G in NP1; G1461A in VP1/VP2) corresponded to patients with significantly lower HBoV1 viral load. Future studies should confirm this putative correlation and investigate the potential implications for clinical care of HBoV1 patients.

**METHODS**

**Samples and clinical information.** NPAs were collected from 846 children who were hospitalized for treatment of RTI in the Department of Respiratory Medicine at the Children’s Hospital of Chongqing Medical University between June 2009 and May 2011. All samples were immediately placed in a chilled transport medium and sent to the testing laboratory, where they were frozen at −80°C within 24 h after collection. All samples were collected with the permission of guardians. Patient medical history was obtained from the guardian. The study protocol was authorized by the Ethics Committee of the Paediatrics College of Chongqing Medical University.

**Nucleic acid extraction and PCR.** Viral DNA and RNA were extracted from 200 μl aliquots of the NPA samples by the QIAamp MinElute Virus Spin kit (Qiagen). The RNA was applied as the template for cDNA synthesis with the SuperScriptII First-Strand Synthesis System (Invitrogen). The DNA extractions and cDNA products were stored at −80°C for subsequent testing of HBoV1 (method described below) and the following common respiratory viruses: IV, PIV, RSV, hMPV and CoV (by nested PCR) (Tiveljung-Lindell et al., 2009); and ADV (by real-time PCR) (Xu et al., 2000).

**Real-time PCR primers and probes.** The HBoV1 NS1 gene-specific primers and probe were used: HBoV1F, 5'-CCATATAAGAGCT-GCTGTACCTTCCTG-3'; HBoV1R, 5'-AAGGATAGCATGACTCAGCACAAG-3'; probe, (FAM)-5'-CCAGAGATGTCTACTCCGGC-3'- minor groove binder (MGB) - BHQ1 (Kantola et al., 2010). The amplified target fragment was cloned into the pMD19-T vector (Takara) and verified by sequencing. Plasmid DNA concentrations were determined by an ND-1000 spectrophotometer (Nanodrop). The real-time PCR was carried out in a total reaction volume of 20 μl consisting of 10 μl of TaqMan Universal Master Mix (Applied Biosystems), 0.6 μl (0.6 μM) of each primer, 0.6 μl (0.3 μM) of probe, 2 μl of template and 6.2 μl of double-distilled water. The real-time PCR thermal cycling reaction and quantitative measurement were performed in a StepOne Real-Time PCR instrument (Applied Biosystems) using the following programme: one cycle of 50°C for 2 min, one cycle of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Each run included plasmid-only and no template negative controls.

The viral DNA extracts from all of the HBoV1-positive cases were sent to Shanghai Majorbio Bio-Pharm Technology for HBoV1 full and partial sequence amplification and sequencing of the PCR products. The primers used for these amplifications are listed in Table 6. Sequence analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA 5) software.

**Statistical analysis.** Data were analysed using the SPSS 17.0 software package. Categorical variables were compared using the χ² test and continuous variables were compared using the Student’s t-test. P-values <0.05 were considered statistically significant.

### Table 6. Primers used in this study to amplify the HBoV1 (GenBank accession no. DQ000496) whole genome and partial gene sequences

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19–3314 nt</td>
<td>F1 (19–36 nt)</td>
<td>TTCCAAATGAGGTCTCGTG</td>
</tr>
<tr>
<td></td>
<td>R1 (3127–3144 nt)</td>
<td>GGTTCACCGTGTATCAAGT</td>
</tr>
<tr>
<td></td>
<td>F2 (2294–2331 nt)</td>
<td>TTGTCCACCAAGAAACG</td>
</tr>
<tr>
<td></td>
<td>R2 (5238–5256 nt)</td>
<td>CATAAGCAAAAAACAGC</td>
</tr>
<tr>
<td>NP1 (3–627 nt)</td>
<td>F1 (3–22 nt)</td>
<td>AGACAGATCGCTCTACA</td>
</tr>
<tr>
<td></td>
<td>R1 (608–627 nt)</td>
<td>AGCGAAAGACGTTCCTCGT</td>
</tr>
<tr>
<td>VP1/VP2 (2–1593 nt)</td>
<td>F1 (3–22 nt)</td>
<td>GCCTCAAATTAAGAGACAGC</td>
</tr>
<tr>
<td></td>
<td>R1 (1574–1593 nt)</td>
<td>CATCCATATGTCGCCGACTA</td>
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</table>
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