Prevalence analysis of different human bocavirus genotypes in pediatric patients revealed intra-genotype recombination

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ABSTRACT

Background: Human bocavirus (HBoV) genotypes 1–4 have been detected worldwide in respiratory samples and stool samples, and are increasingly associated with respiratory and intestinal infections of previously unknown etiology in young children. Several studies revealed evidence of extensive recombination among HBoV genotypes at the NP1 and VP1 gene boundary region. This study explored the prevalence of HBoV genotypes in pediatric patients in Beijing, and studied their phylogeny.
Methods: A total of 4941 respiratory specimens and 1121 fecal specimens were collected from pediatric patients with respiratory infections from January 2006 to December 2013, or with acute diarrhea from October 2010 to December 2012. Conventional PCR was used to detect HBoV1–4 within these samples. Gene fragments at the NP1 and VP1 gene boundary were amplified from HBoV-positive specimens, sequenced, and their phylogenetic inferences constructed using MEGA 6.0 software. Recombination events were identified with SimPlot software.
Results: Human bocavirus 1, 2, and 3 were detected in 9 (0.80%), 15 (1.33%), and 1 (0.08%) of 1121 stool samples, respectively. However, only HBoV1 (82, 1.65%) was detected in respiratory specimens. Phylogenetic analysis of gene fragments at the HBoV NP1 and VP1 gene boundary indicated that HBoV1 sequences obtained from fecal or respiratory specimens across 8 years were highly conserved (99–100%), while 15 HBoV2 sequences collected across 2 years in Beijing were more diverse with up to 4.40% variation. Of the 15 HBoV2 sequences, 14 clustered into a new lineage divergent from other HBoV2 sequences in GenBank. Five HBoV2 genomic sequences were analyzed for recombination, revealing intra-genotype recombination between HBoV2A and HBoV2B.
Conclusions: More HBoV1 were detected in children with respiratory tract diseases, and HBoV2 in patients with acute diarrhea. Phylogenetic analysis revealed a new cluster of HBoV2 was prevalent in China, which may be the result of intra-genotype recombination between HBoV2A and HBoV2B.

1. Introduction

Human bocavirus (HBoV), are members of the genus *Bocavirus* (subfamily *Parvovirinae*, family *Parvoviridae*), and are classified into four genotypes HBoV1–4 based on nucleotide divergence of VP1, or the nonstructural gene and nucleotide capsid region (Kapoor et al., 2010; Koseki et al., 2012; Shen et al., 2013). Since its discovery in nasopharyngeal secretions of children with respiratory tract infections in 2005 by Allander et al. (2005), HBoVs have been identified globally, predominantly in nasopharyngeal aspirates, fecal specimens, cerebrospinal fluid, and blood (Khamrin et al., 2012a,

2012b; Mitui et al., 2012; Mori et al., 2013; Shen et al., 2013; Simon et al., 2007; Tozer et al., 2009; Zhao et al., 2006). HBoV1 was most commonly detected in nasopharyngeal aspirates from children with incidences ranging from 0.8% to 33% (Koseki et al., 2012; Longtin et al., 2008; Martin et al., 2010; Simon et al., 2007; Tozer et al., 2009), while HBoV2–4 were predominantly detected in stool samples (Cheng et al., 2011; Jin et al., 2011). HBoVs are highly prevalent in co-infections with other pathogens, and in asymptomatic children, and to date HBoVs have yet to be confirmed as pathogens. Seroepidemiology of HBoVs has provided more evidence for a causal role of HBoVs in respiratory illness (Jartti et al., 2012).

HBoVs are small non-enveloped viruses containing a single-stranded DNA genome of about 5.3 kb (Cheng et al., 2011; Kapoor et al., 2010; Khamrin et al., 2013). The genome includes

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three ORFs, which encode two non-structural proteins, NS1 and NP1, and two capsid proteins, VP1 and VP2 (Khamrin et al., 2013; Shackelton et al., 2007). HBoV1 is genetically homogeneous, while HBoV2 has a high degree of genetic diversity (Kapoor et al., 2010). In 2010, Kapoor et al. reported homogeneity between the NS1/NP1 genes of HBoV3 and HBoV1, while the HBoV3 VP1/VP2 gene shared similarity with HBoV2. More recently, recombinants originating from HBoV2 and HBoV4 have been reported (Khamrin et al., 2013). It has been reported that the recombination break point was at the NP1 and VP1 gene boundary (Cheng et al., 2011; Kapoor et al., 2010). In the current study, the prevalence, phylogenetic relationship, and recombination events of different HBoV genotypes isolated from pediatric patients in Beijing, China, were explored using polymerase chain reaction (PCR), with primers specific for the NP1/VP1 gene boundary.

2. Materials and methods

2.1. Clinical specimens

During October 2010 to December 2012, 1121 fecal specimens were collected from outpatients (715 were male, 406 were female) who visited the Affiliated Children's Hospital of the Capital Institute of Pediatrics with acute diarrhea, and who experienced three or more loose or liquid stools per day for less than 2 weeks. All stool specimens were diluted (1:10) in phosphate buffer, vortexed, and centrifuged at $1500 \times g$ for 15 min. The supernatants were collected in sterile tubes and stored at $-20\,°C$ before use.

During January 2006 to December 2013, 40–50 respiratory specimens (nasopharyngeal aspirates or swabs) per month were randomly selected from samples submitted for screening of respiratory viruses, collected from hospitalized patients or outpatients diagnosed with respiratory tract infections in the Affiliated Children's Hospital of the Capital Institute of Pediatrics. In total, 4941 respiratory specimens were selected, including 2892 from male patients and 2049 from female patients. The ratio of nasopharyngeal aspirates from hospitalized patients to swabs from outpatients was approximated 7:5. Nasopharyngeal aspirates were processed in 2 mL Hank's buffer (3% ampicillin and kanamycin) and centrifuged at $500 \times g$ for 10 min. The pellets were resuspended in several drops of sterile phosphate-buffered saline and aliquots dispensed on slides and fixed in acetone, before screening for respiratory viruses using the D3® Ultra™ DFA Respiratory Virus Screening & ID Kit (Diagnostic Hybrids, Athens, OH, USA), according to the manufacturer's instructions. The swabs were processed in 2 mL Hank's buffer (3% ampicillin and kanamycin) and centrifuged at $500 \times g$ for 10 min. The supernatants from nasopharyngeal aspirates and swabs were collected in sterile tubes and stored at $-20\,°C$ before use.

The Ethics Committee of the Capital Institute of Pediatrics approved this research work.

2.2. Nucleic acid extraction

DNA was extracted from the supernatants of fecal and respiratory specimens using DNAzol® BD (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions, and the DNA re-suspended in 20 μL 8 mM NaOH.

2.3. Detection of HBoV1–4 by PCR

Published primers HBoV2-sf2 (5′-TGCTTCAA CAGGCAAAACAA-3′) and HBoV2-sr2 (5′-TCCAAGAGGAAATGAGTTTGG-3′) were used to amplify a 495-nt fragment within the ORF of NS1 of HBoV1–4 in clinical specimens (Babkin et al., 2013). The amplification profile

comprised an initial period of denaturation at 94 °C for 5 min, followed by 45 amplification cycles (94 °C for 30 s, 58 °C for 30 s, and 72 °C for 45 s). The amplification products were analyzed by electrophoresis in a 1.5% (w/v) agarose gel. For sequence analysis, HBoV positive samples were screened for the 690-nt fragment at the NP1 and VP1 gene boundary using primers HBoV-c1 (5′-CTTYGAAGAYCTCAG ACC-3′) and HBoV-c2 (5′-TKGAKCCAA TAATKCC AC-3′), designed according to GenBank sequences of HBoV1–4. The amplification profile comprised an initial period of denaturation at 94 °C for 5 min, followed by 45 amplification cycles (94 °C for 30 s, 48 °C for 30 s, and 72 °C for 1 min), with a final 10 min extension at 72 °C. The amplicons were analyzed by electrophoresis in a 1.5% (w/v) agarose gel.

All PCR reactions contained 2.5 μL DNA, 0.5 μL of each forward and reverse primer (10 mM), 0.5 μL Easy Taq DNA Polymerase (TransGen Biotech Co., Ltd., Beijing, China), 2.5 μL 10× Easy Taq Buffer; made up to a final volume of 25 μL with distilled water. The PCR products amplified by the NP1 and VP1 gene boundary primers HBoV-c1 and HBoV-c2 were sequenced, and phylogenetic analysis conducted.

A more thorough evaluation of the phylogenetic relationship and recombination events of HBoV2 was undertaken using nearly full-length genome sequences of HBoV2 Q19, Q390, Q869, and Q952, and primers designed by Zhao et al. (2012).

2.4. Phylogenetic relationship and recombination analysis

Phylogenetic analysis was conducted with MEGA version 6.0 software package. Phylogenetic trees were constructed using the neighbor-joining method and maximum composite likelihood model using sequences of the target gene from HBoV-positive specimens in the study, and HBoV sequences from GenBank. A discrete Gamma distribution, used to model evolutionary rate differences among sites (1 category, +G), was constructed in MEGA 6.0. A bootstrap pre-sampling (1000 replications) was used to assess the reliability of individual nodes in each phylogenetic tree. Recombination potential and localization of recombination break points were evaluated using similarity plot and boot-scanning analyses generated by the SimPlot software package. A total of 33 full-length genome sequences of HBoV1, HBoV2, HBoV3, HBoV4, and porcine bocavirus were downloaded from GenBank, and identified as GenBank No.-HBoV genotype/genus name-country.

2.5. Statistical analysis

The Pearson Chi-Square test was used to evaluate differences in HBoV positivity rates between male and female patients; $p < 0.05$ was considered to be statistically significant. The profiles of HBoV-positive patients of different ages were described using the mean ± standard deviation (SD).

3. Results

3.1. Screening of HBoVs by PCR

Of the 4941 respiratory samples and 1121 stool samples tested, 82 (1.65%) and 25 (2.2%) respectively, were identified as HBoV positive by PCR.

3.2. Phylogenetic analysis of HBoVs

All 107 amplicons of the NP1/VP1 boundary region from HBoV positive specimens (82 from respiratory specimens, 25 from stool specimens) were sequenced and blasted against sequences of HBoV1, HBoV2 (2A and 2B), HBoV3, HBoV4, porcine parvovirus,