Detection of Human Bocavirus from Fecal Samples of Hungarian Children with Acute Gastroenteritis

Katalin N. Szomor^a Beatrix Kapusinszky^a Zita Rigó^a Zoltán Kis^a Mónika Rózsa^a
Ágnes Farkas^a Andrásné Szilágyi^b György Berencsi^a Mária Takács^a

^aDivision of Virology, and ^bDivision of Epidemiology, National Center for Epidemiology, Budapest, Hungary

Key Words

Viral gastroenteritis • Human Bocavirus • Genotypes

Abstract

Objectives: Human bocavirus (HBoV), a newly identified member of the Parvoviridae family is associated with respiratory tract and gastroenteric infections, mostly of young children. HBoV infections show a seasonal distribution with the peak in temperate areas being in the winter months. **Methods:** In our study, 35 throat swabs from children under 5 years with acute respiratory symptoms and 61 stool samples from children (<5 years) with acute gastroenteritis were collected in the period of October 2007–March 2008. A HBoV-specific polymerase chain reaction for detection of the virus, and sequence analysis for identification of virus variants were performed. **Results:** Although respiratory samples were all negative, 3.3% of stool samples (2/61) proved to be positive for HBoV. The virus carrier children were 3 and 5 years old. The ratio of HBoV positive samples is similar to international results (2.1–5.5%). **Conclusions:** According to the result of sequence analysis of HBoV, the occurrence of genotype 2 of HBoV in Hungary is confirmed.

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Introduction

Human bocavirus (HBoV) was identified in 2005 by nonspecific genome amplification methods. Comprehensive studies on sequence and phylogenetic analysis led to classification of the virus to the Parvoviridae family. Due to its close relation to bovine parvovirus and minute virus of canines, the novel parvovirus was named ‘human bocavirus’ [1]. Two variants of the virus have been described [2] so far. However, differences in biological characteristics, disease association, epidemiology and geographical distributions of the genotypes are still poorly understood. HBoV infections show a seasonal distribution, with the peak in temperate areas being in the winter months.

Infections are associated with respiratory diseases and acute gastroenteritis, mostly among young children [3–5]. The virus can be observed by electron microscope in nasopharyngeal secretions of children infected with HBoV [6]. The DNA of the virus has been detected in the blood and nasopharyngeal samples of patients with acute respiratory illness, in the fecal samples of patients with diarrhea [7] (with or without concomitant respiratory symptoms [8]), and – rarely – in urine samples [9]. In a

study based on seroepidemiological results, HBoV infection was found to be common during childhood [10]. Serodiagnostic results (immunofluorescence and ELISA) showed that parvovirus B19 and HBoVs are antigenically distinct since no cross reactivity could be detected [11]. The virus could be isolated in co-infection with other respiratory viruses (human metapneumovirus, respiratory syncytial virus, coronavirus) [12] and enteric pathogens (norovirus, astrovirus, rotavirus, adenovirus 40/41) [13]. HBoV has been detected worldwide, as reviewed by Lindner and Modrow [14]. The presence of HBoV in Hungary has not yet been investigated, so this pilot study aimed to collect data on the presence of the virus in Hungary for the first time. Furthermore, we wanted to determine the genotype of the Hungarian isolates.

Materials and Methods

Samples

Throat swabs and fecal samples were selected from the samples collected in the period of October 2007–March 2008 for our retrospective study. Thirty-five throat swabs were taken from children under 5 years of age with acute respiratory symptoms and 61 stool samples were taken from children under 5 years with acute gastroenteritis.

Viral Diagnostic Procedures Performed Previously

For detection of respiratory pathogens (adenovirus, parainfluenzaviruses, respiratory syncytial virus, influenza A and B viruses, *Chlamydia* and *Mycoplasma pneumoniae*) in the throat swabs, a direct immunofluorescence test by Bartels Virus Respiratory Kit was performed according to the technical protocols of the manufacturer.

For detection of gastroenteric pathogens, we used the rota/adenovirus 40/41 Rota-Adeno Blister immunochromatography test (CerTest Biotec) and the norovirus (calicivirus) Ridascreen Norovirus Third Generation (R-Biopharm) antigen detection ELISA kit. These were used according to the instructions from the manufacturers.

PCR for Sequencing

DNA purification was performed as described previously [15], with phenol/chloroform deproteinization after proteinase K digestion, followed by precipitation by isopropanol. The PCR primer set located on the VP1/VP2 capsid protein coding region of the genome (*HBoV-VPf* 5'-gca aac cca tca ctc tca atg c; *HBoV-VPp* 5'-gct ctc tcc tcc cag tga cat) was used as described previously [16]. The PCR (40 amplification cycles) was carried out in 50- μ l volume using RNase- and DNase-free double distilled water (Gibco), ReadyMix (Sigma) for master mix, 2 μ l of DNA and 40 pmol of primers. The PCR conditions were 94° for 3 min, 94° for 30 s, 54° for 30 s and 72° for 30 s, with a final extension at 72° for 7 min. The PCR products were visualized with ethidium bromide staining and UV lighting after agarose gel-electrophoresis.

Sequencing

For direct sequencing, PCR products were purified with PCR Clean up-M Kit (Viogene). DYEnamic ET dye terminator kit (Pharmacia) was used according to the instructions of the manufacturer. The electrophoresis was carried out on MegaBACE Sequence Analyzer.

Phylogenetic Analysis

330 bp DNA fragments of VP1/VP2 were used to study the phylogenetic relationship of the Hungarian and virus isolates published previously. DNA sequences were aligned using the Multalin software [17]. A phylogenetic tree was constructed by the neighbor-joining method using a Kimura 2-parameter substitution model. To estimate the reliability of the tree topology, a bootstrap analysis of 1,000 replicates was performed using MEGA software (version 3.1) [18].

Restriction Fragment Length Polymorphism

The very few nucleotide polymorphisms of the HBoV genome are located on the capsid protein-coding region. A simple and fast method for differentiation of the virus variants can be the restriction fragment length polymorphism (RFLP). After digestion of the PCR products with the BstAPI endonuclease, the isolates can be divided into 2 groups: genotype 1 (digested by the enzyme) and 2 (remain undigested) [19]. The recognition site of the enzyme is the sequence motif of 'GCANNNNTGC' at nt position 4,820 on the HBoV genome. The recognition motif on sequences of Hungarian isolates was determined in silico by software analysis [20]. RFLP may be a possible tool for further identification of genotypes or for differentiation of virus variants.

Results

Thirteen of 35 (37%) throat swabs of patients under 5 years of age with acute respiratory symptoms proved to be positive for influenza viruses (11/35 influenza A, 2/35 influenza B). Co-infection with other respiratory pathogens including HBoV could not be detected. The respiratory samples were negative for HBoV DNA (table 1).

Sixty-one fecal samples were selected by symptoms and patient age for detection of HBoV. The samples were tested for other gastroenteric viruses such as rotaviruses, adenoviruses and/or noroviruses beforehand. Fifty-two of the 61 samples were also screened for rota- and adenoviruses, 11 of them (21%) proved to be positive for rotavirus. In 2 (3.8%) rota- and adenovirus co-infection could be detected. Forty-seven of the 61 samples were also screened for norovirus, 14.9% (7/47) of them were positive. Thirty-nine of 47 stool samples were tested for rota-, adeno- and noroviruses, no co-infections could be detected. In 2 (3.3%) of the selected 61 fecal samples HBoV could be detected (table 1). The virus carrier children were 3 and 5 years old, their main symptoms were subfebrility, acute enteritis with colica and diarrhea with-

Fig. 1. Phylogram depicting the phylogenetic relationship of the sequences obtained from fecal samples of children with acute gastroenteritis collected in Hungary and previously published sequences representing HBoV genotypes. Percentage bootstrap values greater than 90% are shown at the respective nodes. Typical symptoms of the patients are indicated with abbreviations. ARS = Acute respiratory symptoms; GE = gastroenteritis.

Table 1. Results of serologic and molecular analyses of throat swabs screened for respiratory pathogens and HBoV, and of fecal samples screened for gastroenteric pathogens and HBoV

Pathogen	Sample	Method	Samples, n	Positives, n	Notes
Adeno-, parainfluenza-, respiratory syncytial virus	throat swab	direct immunofluorescence	35	0	
<i>Chlamydia</i> and <i>Mycoplasma pneumoniae</i>	throat swab	direct immunofluorescence	35	0	no co-infection detected
Influenza A	throat swab	direct immunofluorescence	35	11 (31%)	
Influenza B	throat swab	direct immunofluorescence	35	2 (6%)	
HBoV	throat swab	polymerase chain reaction	35	0	
Rotavirus	stool	immunochromatography	52	11 (21%)	2 rota-/adenovirus co-infection
Adenovirus 40, 41	stool	immunochromatography	52	2 (3.8%)	
Norovirus (calici)	stool	antigen detection – ELISA	47	7 (14.9%)	39 rota-/adenovirus tests were performed; no co-infection detected
HBoV	stool	polymerase chain reaction	61	2 (3.3%)	no co-infection detected

out vomiting. No respiratory symptoms had been reported. The illness was mild, the duration of the sickness was 2 days altogether. Both children were kindergarteners and were in different nurseries where gastroenteritis outbreaks had occurred within a week before each child's illness. Bacterial examinations (for *Salmonella*, *Shigella*, *Yersinia enterocolitica*, *Escherichia coli* O124 and *Campylobacter*) of the outbreak produced negative results. The symptoms of the affected children in the kindergarten were similar.

According to the results of sequencing of PCR products followed by phylogenetic analysis, the viruses of the Hungarian children (HBoV-hun1 and HBoV-hun2) belong to the genotype 2, their NCBI GenBank accession numbers are FM209184 and FM209185, respectively (fig. 1). The genotype results were verified by RFLP in silico, when the PCR products were analyzed for the presence of the sequence motif of the BstAPI enzyme digestion site (fig. 2). Hungarian isolates did not have the revealing site, thus PCR amplicons could not be cut by the enzyme, as is the case with other sequences related to this genotype group.

Fig. 2. Comparison of nucleic acid sequences of Hungarian bocavirus isolates with previously published sequences representing different genotypes. Dots indicate identity with the sequence in the first line. Representative sequences of HBoV genotypes are denoted according to their GenBank accession number. Sequences reported in this study are marked by accepted abbreviation of human bocavirus (HBoV), the place of origin (hun) and the number of the patient. The recognition motif (3-3 nucleotides) of the restriction enzyme is shaded in grey.

	nt pos. 4811	4821	4831
HBoV-hun1	g t t c c a a c t	g c c t c a a a t	g c a g a c t c a t a c c
HBoV-hun2	.	.	.
DQ340570	.	.	.
AM849120	.	.	.
EU400128	.	.	.
EF441545	.	.	.
EU069437	.	.	.
DQ839266	.	.	.
EF441550	.	.	.

Discussion

Infectious gastroenteritis is one of the most common diseases in young children. The etiology of diarrhea of ‘non-viral’ gastroenteritis outbreaks remains unidentified in about 25–40% of cases, although the definition ‘non-viral’ could simply mean that virological investigations produced negative results. As we get more and more data about the wide range of viral pathogens which may play a role in acute gastroenteritis the percentage of outbreaks with clarified etiology will increase. The epidemiology and clinical aspects of the newly detected HBoV are poorly understood, but gastroenteritis may be the possible consequence of infection mainly in young children. Samples of 2 children with acute but mild gastroenteritis proved to be positive for HBoV in Hungary, in the winter season of 2007/2008. The children were kindergarteners, where gastroenteritis outbreaks occurred a week before each the child’s illness. No respiratory symptoms had been reported in the day nursery or in either child’s family.

The phylogenetic analysis score and RFLP *in silico* confirmed the occurrence of genotype 2 of HBoV in Hungary (HBoV-hun1, HBoV-hun2). Based on phylogenetic results, no evidence for typical genotype distribution in correlation with symptoms could be detected. The basis of comparison was the sequence collection of virus isolates from patients with different symptoms (both respiratory, AM849120, DQ340570, EU069437, DQ839266, and gastroenteric, EU400128, EF441545, EF441550). Some researchers [7, 21] assume that the virus only passes through the gastrointestinal tract after virus-containing nasopharyngeal secretions are swallowed. However, in the absence of any sign of respiratory illness, and the presence of signs of gastrointestinal infection, the detection of the virus in the fecal sample may indicate a pathogenic role of HBoV in gastroenteritis [14, 22].

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