PRESENCE OF HUMAN BOCAVIRUS 1 IN HOSPITALISED CHILDREN WITH ACUTE RESPIRATORY TRACT INFECTIONS IN LATVIA AND LITHUANIA

Zaiga Nora-Krūkle^{1,#}, Santa Rāsa¹, Anda Vilmane¹, Sabine Grāvelsīņa¹, Mārtiņš Kālis¹, Inga Ziemele^{7,8}, Milda Naciute², Sigita Petraitiene^{3,9}, Diana Mieliauskaite², Migle Klimantaviciene³, Irute Girkontaite², Hsin-Fu Liu⁴, Jih-Hui Lin⁵, Yung-Cheng Lin⁶, Hsiu-Chuan Chan⁶, Dace Gardovska^{7,8}, and Modra Murovska¹

¹ Augusts Kirhenšteins Institute of Microbiology and Virology, Rīga Stradiņš University, Rātsupītes iela 5, Rīga, LV-1067, LATVIA

² Centre for Innovative Medicine, State Research Institute, Žygimantų Str. 9, LT-01102, Vilnius, LITHUANIA

³ Children's Hospital, Vilnius University Hospital Santariškių Klinikos, Santariškių Str. 7, Vilnius, LT-08406, LITHUANIA

⁴ Department of Medical Research, Mackay Memorial Hospital, 92 Chung-Shan North Road, Section 2, Taipei, 10449, TAIWAN

⁵ Center for Research, Diagnostics and Vaccine Development, Centers for Disease Control, No. 161, Kun-Yang Street, Taipei, 11561, TAIWAN

⁶ Institute of Bioscience and Biotechnology, National Taiwan Ocean University, No. 2, Beining Rd, Zhongzheng District, Keelung, 202, TAIWAN

⁷ Children's Clinical University Hospital, Vienības gatve 45, Rīga, LV-1004, LATVIA

⁸ Rīga Stradiņš University, Dzirciema iela 16, Rīga, LV-1007, LATVIA

⁹ Clinic of Children Diseases, Medical Faculty, Vilnius University, Santariškių g. 4, Vilnius, 08406, LITHUANIA

Corresponding author; Zaiga.Nora@rsu.lv

Contributed by Modra Murovska

Human bocavirus 1 (HBoV1) is a parvovirus recently found to be a possible aetiological agent of acute respiratory disease in children. We conducted the first clinical and molecular study on this virus in Latvia (LV) and Lithuania (LT). The aim of the study was to determine the occurrence of HBoV1 in respiratory tract samples taken from hospitalised children with acute respiratory tract infections in LV and LT. In total 186 children with age one to 50 months, and who fulfilled criteria of acute respiratory tract infection, including lower respiratory tract infections, with or without fever, were included in this study. A nasopharyngeal aspirate was obtained from each patient on admission. DNA was isolated and polymerase chain reaction (PCR) performed targeting the HBoV1 NS1 sequence. HBoV1 positive samples were sequenced and phylogenetic analysis was performed. HBoV1 sequence was detected in 42 (32%) of 130 LV and in 8 (14%) of 56 LT samples. In LV the majority of patients with HBoV1 infection were observed in February while in LT in October. The phylogenetic tree for HBoV1 indicated that isolates of HBoV1 cluster closely and include almost all of the isolates in this study. HBoV1 is common in Latvia and Lithuania and might be a significant pathogen that contributes to acute respiratory tract infections in children.

Key words: BoV1, respiratory tract infections, phylogenetic analysis.

INTRODUCTION

Lower respiratory tract infections (LRTIs), which are frequently caused by viruses, are one of the leading causes of morbidity and mortality in children worldwide (Woensel *et al.*, 2003). Every year in the United States approximately 30 000 children are hospitalised during their first year of life due to viral lower respiratory tract infections (Shay *et al.*, 1999). Moreover, in developing countries each year up

to 1.8 million children die due to acute respiratory diseases (Mathers *et al.*, 2008). Only about 40% of lower respiratory tract viral illnesses have an aetiological agent (Kahn, 2007). Also in Latvia and Lithuania there is a serious health care problem regarding respiratory tract diseases in the children population. Data from the Disease Prevention and Control Centre show that respiratory tract diseases in children under one year of age take the first place in the structure of morbidity. It is also important that during the

last four years there have been more cases not only with acute upper respiratory tract infections but also with lower respiratory tract infections.

Human bocavirus (HBoV) was first discovered in 2005 by Tobias Allander and colleagues in Sweden in pooled respiratory samples from children with respiratory tract infection (Allander *et al.*, 2005). HBoV belongs to the *Parvoviridae* family, *Parvovirinae* subfamily, *Bocaparvovirus* genus (Yoo *et al.*, 2015). *Parvoviridae* family members are small, non-enveloped viruses with isometric nucleocapsids (diameters of 18 to 26 nm), which contain a single molecule of linear, negative-sense or positive-sense, single-stranded DNA. The complete genome size of HBoV1 is 4000 to 6000 nucleotides. Three open reading frames are found in the genome. Of them, two encode non-structural proteins (NS1 and NP1) and one encodes two capsid proteins (VP1 and VP2) (Allander *et al.*, 2005).

So far four types of HBoV are known (Allander *et al.*, 2005; Kapoor *et al.*, 2009; 2010). HBoV type 1 (HBoV1) is a respiratory virus and has been associated with both acute upper and lower respiratory tract infections (Allander *et al.*, 2007; Söderlund-Venermo *et al.*, 2009; Christensen *et al.*, 2010). HBoV1 has a worldwide distribution and has been detected in Europe, Africa, Asia, North America, Australia, and the Middle East. No racial and gender predilection is known. The prevalence of HBoV1 in respiratory tract secretions ranges from 1.5 up to 22% (Bastien *et al.*, 2006; Li *et al.*, 2013). However, one study showed prevalence of HBoV1 in respiratory tract secretions to be as high as 59% of patients (Martin *et al.*, 2010).

So far, the occurrence of HBoV1 has not been investigated in Latvia and Lithuania. Taking into account the high morbidity of acute respiratory tract diseases in children, it is important to determine the occurrence of HBoV1 infection in Latvia and Lithuania.

Thus, the aim of this study was to determine the occurrence of the HBoV1 genomic sequence in respiratory tract samples taken from hospitalised children with acute respiratory tract infections in Latvia and Lithuania. Sequences of HBoV detected in the present study were further characterised for their genetic evolutionary relationships with the viruses circulating in this area and HBoV reference strains. The phylogenetic analysis was conducted by our colleagues from Taiwan.

MATERIALS AND METHODS

Study subjects and inclusion criteria. The study was performed in the Children's Clinical University Hospital of Riga, Latvia, from November 2012 to June 2015 (there were no patients in summer months due to lack of hospitalised patients with LRTIs) and in the Children's Hospital, Affiliate of Vilnius University Hospital Santariskiu Klinikos, Lithuania, from September 2013 to March 2014. Children aged one to 50 months who were hospitalised in

the Paediatric Department and fulfilled the criteria of acute respiratory tract infection (ARTI), including LRTI, with or without fever, were included in this prospective study. Fever was defined as a core temperature ≥ 38 °C. LRTI was defined according to the clinical definition criteria and/or chest roentgenogram with positive results (infiltrates). According to the clinical definition of LRTI, at least one specific lower respiratory tract sign from the following (Roth *et al.*, 2008) should be positive: fast or difficulty in breathing; chest wall in drawing; abnormal auscultator findings (crackles/crepitation or bronchial breath sounds).

On inspection, fast breathing or tachypnea was defined based on the World Health Organisation (WHO) criteria: respiratory rate > 60 per minute in children aged < 2 months, > 50 per minute in children aged 2–12 months, and 40 per minute in children aged 12–60 months (Anonymous, 1995).

A total of 130 children [74 (57%) male and 56 (43%) female, with mean age 15 ± 11 months (range 1 to 50 months)] from Latvia and 56 children [40 (70%) male and 16 (30%) female, with mean age 25 ± 14 months (range 4 to 48 months)] from Lithuania were included in this study.

The study protocol was approved by the Ethics Committee of the Rīga Stradiņš University and by the Ethics Committee for Vilnius Regional Biomedical Research. Written informed consent was obtained from the parents of all participating children.

Laboratory methods. All clinical samples were tested in the corresponding home country according to a previously agreed work plan using common methods.

Human DNA was extracted from 187 nasopharyngeal aspirates (NPAs) by phenol-chloroform extraction. The quantity of DNA was measured spectrophotometrically, and the quality of DNA was tested using β -globin gene polymerase chain reaction (PCR). The HBoV1 NS1 gene sequence was identified in extracted DNA using a qualitative PCR method with HBoV1 specific primers (Sloots *et al.*, 2006) with subsequent electrophoretic analysis of PCR products in 1.5% agarose gel.

Sequencing of HBoV1 specific PCR product. HBoV1 was confirmed in NPAs DNA by sequencing of PCR products. The Latvian researcher group analysed the samples according to the following protocol: VP1 gene sequencing reactions were performed using a Big Dye Terminator kit (Applied Biosystems, USA) and the ABI 3100, sequencer. Nested PCR primers (Kapoor *et al.*, 2010) were used (5'-3'): AK-VP-F1, CGCCGTGGCTCCTGCTCT and AK-VP-F2, TGTTCCGATCACAAAAGATGTG for the first round of amplification, AK-VP-R1, GGCTCCTGCTCTAGGAAATAAAGAG and AK-VP-R2, CCTGCTGTTAGGTCGTTGTTGTATGT for the second round of amplification and for sequencing of both amplified DNA strands.

PRIMERS FOR HBoV1 CODING SEQUENCES ANALYSIS

Target	Primer	Orientation	Sequence (5' → 3')
Fragment A (NS1)	F161	Forward	CCA CGC TTG TGG TGA GTC TA
	R1054	Reverse	GCA TGC CCA AGA CTT GTT CT
Fragment B (NS1)	F1060	Forward	GCC TAG CTG CGT CTT CTG TT
	R2208	Reverse	GTC TCA GGC TCG GTG TCT TC
Fragment C (NP1)	F2255	Forward	CCA GCA AGT CCT CCA AAC TC
	R3094	Reverse	CGC GAT CAG CGT TAT TTA CA
Fragment D (VP1/VP2)	HBo VP1/VP2	Forward	GAT AAC TGA CGA GGA AAT G
	HBo VP1/VP2	Reverse	GAG ACG GTA ACA CCA CTA
Fragment D1 (VP1/VP2)	HBoV-D1	Forward	AGATGTAATTACTGGGATGATGTG
	HBoV-D1	Reverse	GATGTGAACGCCAGCTGTGAGGTC
Fragment D2 (VP1/VP2)	HBoV-D2	Forward	GCAGCGCCTTACAAATGAATA
	HBoV-D2	Reverse	CATCGGGCTGTGGTCTTGAAC

Fig. 1. Localisation of PCR products that were used for sequencing of the complete HBoV genome. NS1, NP-1, VP1 and VP2 — DNA sequences that encode corresponding virus proteins. A, B, C, D, D1, D2 — PCR products that were cloned and used for sequencing.

In Lithuania, sequencing of the PCR product was conducted with another protocol using primers A, B, C, D, D1, and D2 (Fig. 1, Table 1).

PCR products were purified from the agarose gel using a GeneJET™ Gel Extraction Kit, cloned into pJET 1.2 vector using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, Lithuania) and transformed into *E. coli* DH5a cells. Plasmid DNA was isolated and tested by restriction digestion.

The sequencing was performed in BaseClear company (Netherlands) directly from PCR products using the same primers as for PCR or from plasmids using the pJET1.2 forward and reverse sequencing primer.

Phylogenetic analysis. Thirty-one strains of HBoV partial VP1 gene (487bp) from Taiwan, Latvia, and Lithuania were used to construct a phylogenetic tree. VP genes were aligned by Muscle implemented in the MEGA 6 software (Tamura *et al.*, 2013). To ensure the consistency of tree topologies, the phylogenetic trees were reconstructed with the neighbour-joining (NJ) and maximum likelihood (ML) methods using MEGA 6 and PhyML 3.0 (Guindon *et al.*, 2010; Tamura *et al.*, 2013). The robustness of the phylogenetic trees was statistically evaluated by bootstrap analysis with 1000 replicates. A bootstrap value > 75% was considered to represent a monophyletic group.

Statistical analysis. The statistical analysis was performed using GraphPad Prism 6.0. software. Fisher's exact test was

performed to assess the significance of differences. A *p* value < 0.05 was considered statistically significant. For the analysis of patient data, descriptive statistics were used.

RESULTS

A total of 186 patients were enrolled in the study. The mean age for patients from Latvia (LV) was 15 ± 11 months and for patients from Lithuania (LT) — 25 ± 14 months. Forty-two (32%) of the 130 patients (74 male and 56 female) who were recruited in LV were found to be positive for HBoV1 by PCR and subsequent sequencing. Of the 42 positive patients, more were males (62%) than females (*p* = 0.048).

In order to assess HBoV1 prevalence among age groups, we divided the patients into four groups: 1st group: 0–6 months (*n* = 29; 19 male and 10 female), 2nd group: 6–12 months (*n* = 28; 12 male and 16 female), 3rd group: 12–24 months (*n* = 48; 29 male and 19 female) and 4th group: 24–60 months (*n* = 25; 14 male and 11 female). There were significantly more patients in the 3rd group in comparison to the 1st (*p* = 0.014), 2nd (*p* = 0.009) and 4th group (*p* = 0.002).

Of the 29 patients in the 1st age group, the HBoV1 sequence was detected in NPAs DNA samples in four male and three female patients; from 28 patients in the 2nd age group — in seven male and four female NPAs DNA. Peak prevalence of HBoV1 infection was observed in patients ranging from 12 to 24 months (eight male and seven female patients of 48 patients, respectively). In the 4th age group, the HBoV1 sequence was detected in seven male and two female patients of 25 patients in total. There was no statistical difference between distribution by gender in age groups of positive patients. Age distribution of patients with and without presence of the HBoV1 genomic sequence in NPAs DNA as a percentage is shown in Figure 2.

The HBoV1 genomic sequence was detected in samples obtained throughout the year (excepting summer) with the detection rate being the highest in February (21/42, 50%), followed by September (5/42, 11.9%) and January (5/42, 11.9%) (Fig 3).

Fig. 2. Age distribution of patients with and without presence of HBoV1 in Latvia.

Fig. 3. Monthly distribution of HBoV1 infection prevalence in Latvia.

Of the 56 patients from LT, significantly more ($p = 0.0001$) were male ($n = 40$) than female ($n = 16$).

Eight patients of 56 (14%) were positive for HBoV1 sequence in NPAs DNA. Of these eight patients, there was no significant difference in distribution male ($n = 5$) and female ($n = 3$) patients.

Patients from LT were divided into age groups: 1st group: 0–6 months ($n = 6$; 4 male and 2 female), 2nd group: 6–12 months ($n = 6$; 5 male and 1 female), 3rd group: 12–24 months ($n = 19$; 17 male and 2 female) and 4th group: 24–60 months ($n = 25$; 14 male and 11 female). The size of the 4th and 3rd groups in comparison to the 1st and 2nd group was significantly larger ($p = 0.0001$ and $p = 0.0057$, 4th versus 1st and 3rd versus 2nd, respectively).

The prevalence of HBoV1 infection per age group in children aged 0–60 months was as follows: 1 female of 6 patients in the 1st group, none in the 2nd group, 1 female and 3 male among 19 patients in the 3rd group and 1 female and 2 male among 25 patients in the 4th group. The age distribution of patients with and without presence of the HBoV1 genomic sequence in NPAs DNA as percentages is shown in Figure 4.

Researchers from LT obtained samples only from September 2013 till March 2014. The highest prevalence of HBoV1 infection in patients from Lithuania was in October (37.5% of all positive patients) following by September and January (25% of positive cases) (Fig 5).

HBoV isolates from LV and LT were sequenced to perform phylogenetic analysis using the partial VP coding region. The phylogenetic tree for HBoV indicated that isolates of HBoV1 clustered closely. The gene diversity of HBoV1 isolates was significantly low (0–0.9%) between Taiwan, Latvia and Lithuania (Fig. 6).

DISCUSSION

This is the first report on prevalence of HBoV1 in Latvia and Lithuania. The proportion of HBoV1-positive cases among children with acute respiratory tract infection, including lower respiratory tract infection, was 14% in Lithuania, which is similar to that reported in Spain, where 17.8% of children with pneumonia were HBoV positive (Garcia *et al.*, 2018) and Norway, where 25% of children with acute respiratory tract infections were positive for HBoV (Christensen *et al.*, 2013). Our colleagues from Tai-

Fig. 4. Age distribution of patients with and without presence of HBoV1 in Lithuania.

Fig. 5. Monthly distribution of HBoV1 infection prevalence in Lithuania.

wan in their first report on HBoV infection in Taiwan also reported that nearly 10% of children less than two years old with LRTIs were positive for HBoV. Results from Taiwanese colleagues reflect percentage of HBoV positive patients with no co-infections (Jih-Hui *et al.*, 2009). However, HBoV1 was widespread in Latvia (32%). We have no evidence of co-infection, and further studies are required to determine if other viruses or bacteria can cause acute respiratory tract infections, including LRTIs. However, we expect that in the HBoV1 positive cases there was more than one infectious agent. One of the reasons for the relatively high proportion of HBoV1 positive cases in Latvian patients might be due to the selection criteria of children with ARTIs including LRTIs. Martin and co-authors, in 2010, reported that HBoV occurred in 33% cases of ill children, but that presence of the virus was not associated with the presence of a respiratory illness or with specific respiratory symptoms (Martin *et al.*, 2010).

In our study we showed that in the LV group of patients with HBoV1 there were significantly more male (62%) than female ($p = 0.048$) patients. In another study, 73.1% of male

patients with respiratory symptoms were found to be positive for HBoV (Jang Su *et al.*, 2011). However, there was no significant difference in gender distribution among patients positive for HBoV1 in the LT group ($p = 0.619$).

Among HBoV1 positive children, in LV and in LT the majority of patients were 12 to 24 month old (35.7% and 50% of positive cases, respectively). Other studies have shown that HBoV is found in patients 5–6 months of age and older (Allander *et al.*, 2005; Sloots *et al.*, 2006); some authors suggested that maternal antibodies might prevent neonatal infection with HBoV (Ma *et al.*, 2006; Garcia-Garcia *et al.*, 2007). However, we identified HBoV1 also among infants younger than 3 months of age, suggesting incidence of HBoV infection very early in life. In the USA, HBoV has been detected in children as early as a few days after birth (Ghietto *et al.*, 2012) and a seroepidemiological study also provided evidence that HBoV infection is common during early infancy (Kahn *et al.*, 2008).

HBoV1 infection peak prevalence in LV and LT groups slightly differed. In LV the majority of patients with

Fig. 6. Phylogenetic tree of human bocavirus based on partial analysis of VP gene. Phylogenetic tree was constructed using the maximum likelihood method. Only posterior probabilities values above 95% are shown. Abbreviation TW represents the HBoV1 isolated from Taiwan; Latvia represent the HBoV1 isolated from Latvia; Lithuania – the HBoV1 isolated from Lithuania.

HBoV1 were hospitalised in February while in LT in October. Both of these months are in cold season when we can expect more patients with ARTIs or LRTIs. The HBoV positive cases occurred from autumn through spring. There were no samples taken in our study during summer months due to lack of hospitalised patients with acute respiratory tract infections. The seasonal pattern of HBoV occurrence has been previously reported (Kesebir *et al.*, 2006; Sloots *et al.*, 2006) although some studies found that HBoV occurs

throughout the year with a spring outbreak (Bastien *et al.*, 2006).

Within the small region of study, the phylogenetic analysis indicated close relationship between the Latvian and Lithuanian HBoV isolates. The isolated viruses from Latvia are phylogenetically close not only to some Swedish and Lithuanian isolates, but also to Taiwanese isolates, indicating the global nature of HBoV1 distribution.

At present, there is little information on the role of HBoV1 in the aetiopathogenesis of respiratory tract diseases or disease severity depending on HBoV1 loads and co-infection. However, the stable rate of HBoV1 infection in autumn and winter indicates that HBoV1 is an important pathogen for hospitalised children suffering from acute respiratory tract infections, including lower respiratory tract infections.

Further investigation should be conducted to examine the importance of HBoV1 and other co-infecting viruses on respiratory tract diseases.

ACKNOWLEDGEMENTS

This study was supported by Republic of China (Taiwan) – Republic of Latvia – Republic of Lithuania scientific collaboration project, “Establishing of the framework to track molecular epidemiology of Parvoviruses and to correlate sequence variability with different clinical manifestations” (Research Council of Latvia Nr. gr. 6-25/2012/0026, Research Council of Lithuania TAPLLT02/201) and by project Nr. RSU ZP 17/2013 “Epidemiology, pathogenicity of human Bocavirus (HBoV) species and possible association with lower respiratory tract illnesses and acute gastroenteritis in children”. We are grateful to Rita Nikitenkiene and Irina Maksimova for technical help.

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Received 2 November 2015

CILVĒKA BOKAVĪRUSA 1 KLĀTBŪTNE LATVIJĀ UN LIETUVĀ HOSPITALIZĒTIEM BĒRNIEM AR AKŪTĀM ELPCEĻU SLIMĪBĀM

Pirmā tipa cilvēka bokavīruss (HBoV1) ir parvovīruss, kas tiek uzskatīts par iespējamu etioloģisku faktoru akūtu respiratoru slimību gadījumā bērniem. Mēs veicām pirmo klīnisko un molekulāro pētījumu par HBoV1 infekcijas sastopamību Latvijā (LV) un Lietuvā (LT). Raksta mērķis bija noskaidrot HBoV1 klātbūtni respiratorā trakta paraugos, kas iegūti no hospitalizētiem bērniem ar akūtām elpceļu slimībām LV un LT. Pētījumā iekļauti 186 hospitalizēti bērni vecumā no 1 līdz 50 mēnešiem ar akūtām elpceļu slimībām. Tika iegūts nazofaringeālā aspirāta paraugs bērna pirmajā stacionēšanas dienā, izolēta DNS un noteikta HBoV1 NS1 sekvenču klātbūtne, lietojot polimerāzes ķēdes reakciju. Pozitīvie HBoV1 paraugi sekvencēti un veikta filoģenētiskā analīze. HBoV1 konstatēja 42 (32%) no 130 LV un 8 (14%) no 56 LT paraugos. Latvijā HBoV1 biežāk atrada paraugos, kas ņemti februārī, bet Lietuvā — oktobrī. Filoģenētiskā analīze parādīja, ka HBoV1 izolāti ir cieši radniecīgi viens otram. HBoV1 cirkulē Latvijā un Lietuvā un, iespējams, ir nozīmīgs patogēns, kas iesaistīts akūtu elpceļu infekciju izraisīšanā bērniem.