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Human Bocavirus in Nasopharyngeal Secretion of Hospitalized Children with Acute Respiratory Tract Infection – First Year Results of a Four-Year Prospective Study

Humani bokavirus u nazofaringealnom sekretu hospitalizirane djece s akutnom infekcijom dišnog sustava - Rezultati prve godine četverogodišnjeg prospektivnog istraživanja

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Abstract

Background. Human bocavirus (HBoV) is a recently discovered parvovirus that may cause respiratory disease. The aim of this study was to determine HBoV prevalence among hospitalized children with acute respiratory tract infection (ARI) in two Croatian hospitals, Children's Hospital Zagreb and General Hospital Karlovac, and to compare it with prevalence of other respiratory viruses.

Methods. From May 2017 to April 2018 nasopharyngeal and pharyngeal swabs from a total of 275 children with ARI of suspected viral etiology were obtained and tested by multiplex- PCR for the presence of 15 respiratory viruses, including HBoV.

Results. Viral etiology was proved in 221/275 (80.4%) of the patients. HBoV was detected in 17 (6.2 %) samples. Two thirds of HBoV positive patients were between one and three years of age. HBoV was detected in older children when compared to the children infected with respiratory syncytial virus ($P < 0.001$), but younger when compared to those infected with influenza ($P = 0.009$). Seventy six percent of HBoV positive patients had upper and 24% lower respiratory tract infection. HBoV was more often detected in multiple virus infections compared to the other respiratory viruses ($P < 0.001$). HBoV is detected throughout the year except for the summer months.

Conclusion. Human bocavirus, although often detected in combination with other respiratory viruses, is one of the possible causes of ARI in children. Continuous laboratory detection of HBoV in the respiratory secretion of children with ARI is required in order to complete our findings on clinical significance and epidemiology of this infection.

Sažetak

Uvod. Humani bokavirus (HBoV) je nedavno otkriveni parvovirus koji može uzrokovati bolest dišnog sustava. Cilj ove studije je odrediti prevalenciju HBoV u djece primljene zbog akutne respiratorne infekcije (ARI) u dvije hrvatske bolnice, u Kliniku za dječje bolesti Zagreb i Opću bolnicu Karlovac te usporediti s prevalencijom drugih respiratornih virusa.

Metode. Od svibnja 2017. do travnja 2018. testirano je ukupno 275 obrisaka nazofarinksa i ždrijela prikupljenih od djece s ARI sa sumnjom na virusnu etiologiju. Uzorci su testirani metodom multipleks-PCR na prisutnost 15 respiratornih virusa uključujući i HBoV.

Rezultati. Virusna etiologija je dokazana u 221/275 (80,4%) bolesnika. HBoV je detektiran u 17 (6,2 %) kliničkih uzoraka. Većina djece s HBoV infekcijom (70,2 %) bila je u dobi između jedne i tri godine. Djeca u kojih je detektiran HBoV bila su starija od djece inficirane s respiratornim sincicijskim virusom

($P < 0.001$), ali mlađa od djece inficirane virusom influence ($P = 0.009$). Tri četvrtine HBoV inficirane djece imalo je dijagnozu infekcije gornjeg dišnog sustava (76%), dok je 24% djece imalo infekciju donjeg dišnog sustava. HBoV je značajno češće dokazan u koinfekciji s nekim od ostalih respiratornih virusa u odnosu na druge respiratorne viruse ($P < 0.001$). Virus je detektiran tijekom cijele godine, izuzev ljetnih mjeseci.

Zaključak

Humani bokavirus, iako često detektiran u kombinaciji s drugim respiratornim virusima, jedan je od mogućih uzročnika ARI u djece. Potrebna je kontinuirana laboratorijska detekcija ovog virusa u respiratornom sekretu djece s ARI kako bismo upotpunili saznanja o kliničkom značenju i epidemiologiji ove infekcije.

Introduction

Human bocavirus (HBoV) is a recently discovered parvovirus that can be present in the respiratory tract of patients with acute respiratory infection (ARI), as well as in the gastrointestinal tract of patients with gastroenteritis, but its etiologic involvement in the underlying diseases is still uncertain. The virus was discovered in 2005 in Sweden by Tobias Allander et al. [1], using a system of genomic virus screening in nasopharyngeal samples of 17 children with acute respiratory disease. The newly identified virus shared sequence similarities and genomic organization with the bovine parvovirus and a minute virus of canines and was named human bocavirus 1 (HBoV 1) [2]. Later, three additional types were characterized and named HBoV 2–4. To this date, HBoV is classified into *Parvoviridae* family and *Bocaparvovirus* genus, with two species that infect humans: *Primate bocaparvovirus* 1 that contains HBoV types 1 and 3, and *Primate bocaparvovirus* 2 containing HBoV types 2 and 4 [3].

Viral structure of HBoV is typical of *Parvoviridae*, that is, non-enveloped capsid of icosahedral symmetry and diameter of ~25nm. Viral genome is small linear single-stranded DNA, size only 5 kb in length with still unknown terminal sequences. Thereby, viral replication cycle is highly dependent on cellular functions, including DNA polymerase. The genome putatively encodes two forms of the nonstructural protein NS1 and, for bocaviruses unique, nuclear phosphoprotein NP1, as well as two major structural proteins, VP1 and VP2 [4,5].

HBoV 1 infects only well- differentiated or polarized primary human airway epithelial cells. After several attempts, virus was finally successfully grown in cell culture [6,7], but the pathogenesis of HBoV still remains poorly characterized, mainly due to difficulties in replicating the virus in *in vitro* cell cultures and the lack of experimental animal models.

Clinical significance of human bocaviruses has been studied over past decade, and it is generally accepted that HBoV 1 is associated with respiratory tract infections. Pneumonia, bronchiolitis, acute otitis media, the common cold, and exacerbations of asthma are the most common clinical manifestations of HBoV 1 respiratory tract infections, with cough, fever, rhinitis, and wheezing being the most prominent symptoms [8-17]. Primary infection occurs early in life, similar as for other respiratory viruses, such as respiratory syncytial virus (RSV), rhinoviruses and human metapneumovirus. By the age of 6 the HBoV 1-specific IgG seroprevalence is 80% [18,19], still, due to incomplete immunity or falling immune status in old age, symptomatic reinfections can occur. Other three types (HBoV 2-4) have been detected in stool samples but their association with gastrointestinal illness is controversial [20,21]. HBoV 1-4 have been also identified in samples other than respiratory and gastrointestinal tract. Besides respiratory tract, HBoV 1 can be found in serum of symptomatic patients with ARI, pointing to a systemic spread [8], but also in asymptomatic healthy individuals. Namely, it has been detected in up to 5% of pooled human plasma thus making possible concern in transfusion medicine [22,23]. Additionally, it has been identified in cerebrospinal fluid of children with encephalitis in a few cases [24,25].

Due to the difficulties in replicating the virus in cell cultures, the diagnosis of HBoV infection is exclusively based on molecular detection methods [4,10]. Most laboratories currently use in-house PCR and real-time PCR assays targeting the NP-1, NS-1 or VP1/2 gene, but other nucleic acid-based detection methods for the diagnosis of HBoV have also been described. A few commercially available approved multiplexing assays for respiratory viruses that have included HBoV detection have been developed and placed to the market [26].

The aim of this report is to present results of the continuing one-year HBoV PCR detection in nasopharyngeal samples of children with ARI, hospitalized in two Croatian hospitals, Children's Hospital Zagreb and General Hospital Karlovac.

Material and methods

Patients and samples

The samples to be screened were obtained from 275 children consecutively admitted to the Children's Hospital Zagreb and General Hospital Karlovac in the period from May 2017 to April 2018. The samples for molecular diagnostics on respiratory viruses were collected in children presented with symptoms of ARI, in whom viral etiology was suspected (according to the clinical manifestation and biochemical parameters - i.e. normal or slightly elevated white blood cells and/or C- reactive protein). Nasopharyngeal and pharyngeal swabs were collected using flocked

swab, combined and placed into viral transport medium (UTM™, Copan Italy). The swabs were transported at +4°C to the Laboratory within 48 hours were they were stored at −80°C until tested. Specimens were accompanied with demographic data and clinical diagnosis.

The study was approved by the Ethics Committee of the Teaching Institute of Public Health „Dr. Andrija Štampar". Written consent was obtained from the children's parents or caretakers.

Laboratory diagnosis

Detection of HBoV was performed by real time RT-PCR and it included three major steps: nucleic acid isolation, target amplification and detection of product. To isolate viral DNA and RNA from viral transport medium, 300 μL was extracted according to the manufacturer's protocol using Ribospin™ vRD kit (Gene All Biotechnology, Seoul, Korea). Target amplification was done by using multiplex one-step RT-PCR assay, Seeplex® RV15 OneStep ACE Detection (Seegene, Seoul, Korea) on thermal cycler GeneAmp® 9700 PCR System (Applied Biosystems, Foster City, USA) which simultaneously amplifies target sequences of 15 respiratory viruses: influenza A virus, influenza B virus, RSV type A, RSV type B, human adenovirus, human metapneumovirus, human coronavirus 229E/NL63, human coronavirus OC43, human parainfluenza virus 1,2,3 and 4, human rhinovirus A/B/C, human enterovirus and human bocavirus 1/2/3/4. Seeplex® RV15 Onestep ACE Detection assay includes two internal controls: PCR control and whole process control to ensure that PCR worked properly. Products of amplification were detected by microchip electrophoresis detection on MCE®-202 MultiNA device (Shimadzu, Kyoto, Japan) including software analysis displaying the results in form of electropherogram and virtual gel [27].

Statistical analysis

Comparison between groups was performed using the Chi-square test, and statistical software on which all calculations were made was "R". $P < 0.05$ was considered significant.

Results

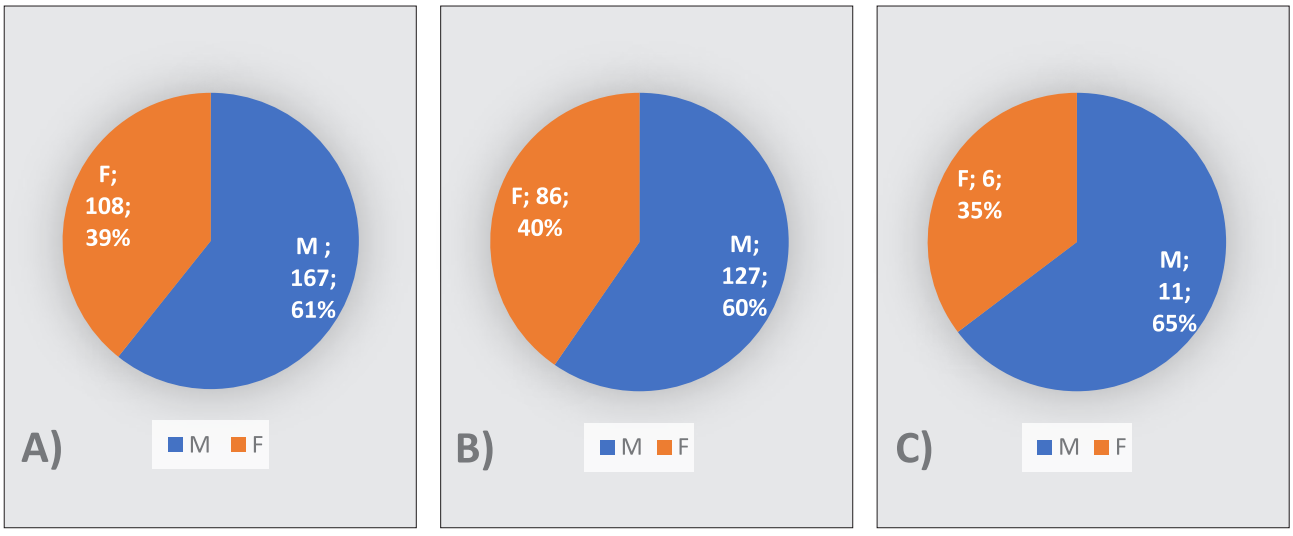
Among 275 tested children there were 167 boys and 108 girls. Gender distribution of tested patients, patients positive for any respiratory virus and patients positive for HBoV is shown in Figure 1. Ninety-nine of the tested patients were younger than one year, 78 were one to three years, 35 were three to five years and 63 of them were older than five years (Figure 2). Temporary distribution of collected and tested samples with the indicated rate of samples positive on any respiratory virus is shown in Figure 3.

Viral etiology was proven in 221 /275 (80.4%) patients. Most frequently detected virus was rhinovirus, which was detected in 105 (38.2%) samples (in 63 samples as single virus - monoinfection), following RSV A/B in 51 sample (18.5%) (monoinfection in 26 samples) and adenovirus in 49 (17.8%) samples (monoinfection in 11 samples) (Figure 4).

Human bocavirus was detected in 17 samples (6.2%) from 11 males and six females. (Figure 1.) Among them the youngest child was two months old and the oldest was 51 months, and average age of HBoV infected children was 21 months. Most children with HBoV infection (70.2%) were between the ages of one and three years (Figure 5), as opposed to the children infected with other

Figure 1. Distribution of tested patients by gender: a) all tested patients, b) all patients with sample positive for any respiratory virus, c) patients with sample positive for human bocavirus (HBoV)

Slika 1. *Raspodjela ispitanika po spolu a) svi testirani pacijenti, b) pacijenti s uzorkom pozitivnim na respiratorne viruse, c) pacijenti s uzorkom pozitivnim na humani bokavirus (HBoV)*



respiratory viruses that were mostly up to 1 year old (P = 0.002). HBoV was detected in older children when compared to the children infected with respiratory syncytial virus ($P < 0.001$), but younger when compared to those infected with influenza ($P = 0.009$).

Thirteen children with detected HBoV had infection of upper respiratory tract infection (URTI) and four children had lower respiratory tract infection (LRTI) (Figure 6). There was no significant differences in localization of infection (URTI vs. LRTI) when comparing HBoV positive patients with other respiratory virus positive patients ($P = 0.08$). In five samples, only HBoV pathogen was detected, thus coinfection with other respiratory viruses was found in 12 samples, including detection of rhinovirus, adenovirus, coronavirus, RSV type B and enterovirus (Figure 7 and Figure 8). HBoV is significantly more often

Figure 2. Age group distribution of all tested patients with acute respiratory infection (ARI) during May 1st, 2017- April 30th 2018 (N=275)

Slika 2. *Raspodjela svih ispitanika po dobi u razdoblju od 1. svibnja 2017. do 30. travnja 2018. (N=275)*

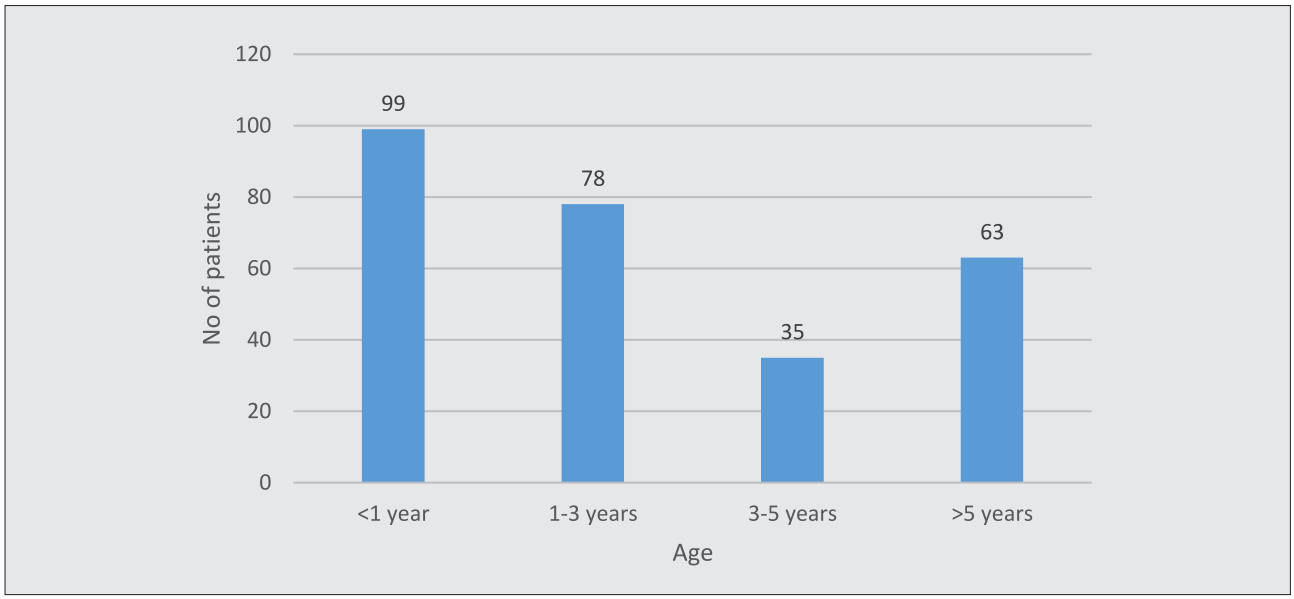
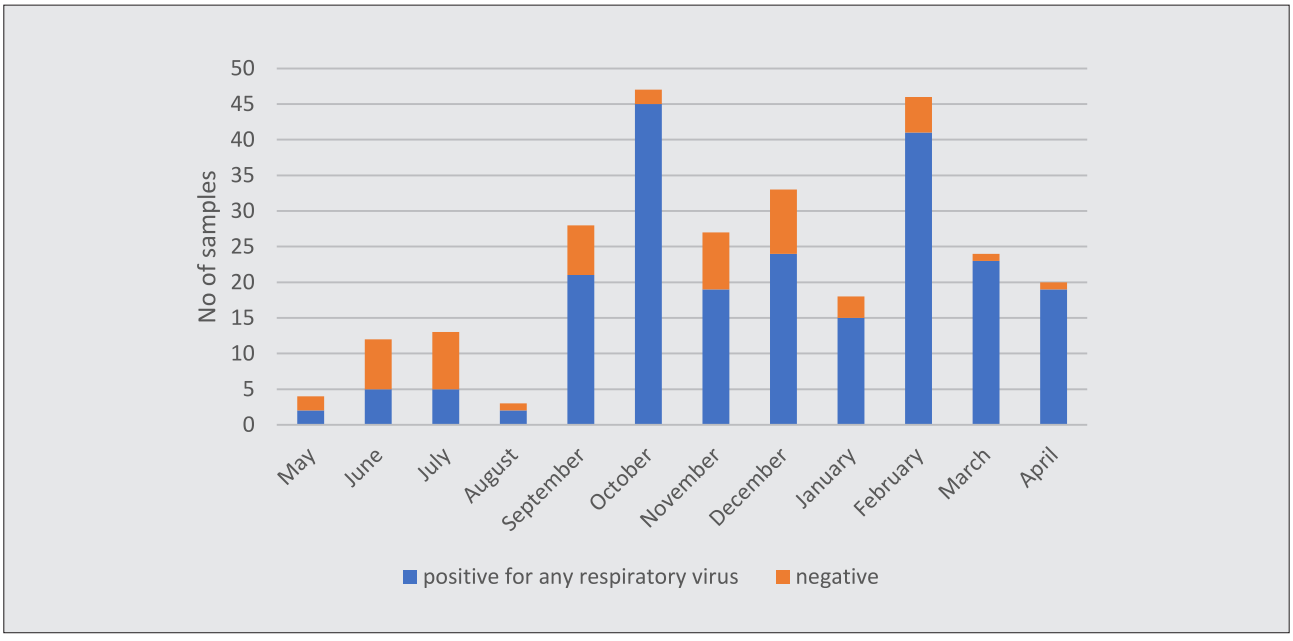


Figure 3. Number of collected and tested samples (May 1st, 2017- April 30th 2018); N=275

Slika 3. *Broj prikupljenih i testiranih uzoraka po mjesecima (od 1. svibnja 2017. do 30.travnja 2018.); N=275*



detected together with other respiratory virus when compare to the other respiratory viruses ($P < 0.001$).

In one child with HBoV positive sample, bacterial infection was confirmed and BHS-A from pharyngeal swab was detected. Four HBoV positive children received empiric antibiotic therapy. None of 17 HBoV positive children needed mechanic ventilation or oxygen therapy. The average duration of hospitalization of HBoV positive patients was 5.7 days. HBoV is detected throughout the year, except for the summer months (Figure 9).

Figure 4. Frequency of detected viruses in samples with positive multiplex PCR for respiratory virus (HRV= human rhinovirus A/B/C, RSV A/B= respiratory syncitial virus A and B, AdV= adenovirus, HCov OC43=human coronavirus OC43, Flu A/B=influenza A and B virus, HBoV= human bocavirus 1/2/3/4, HEV= human enterovirus, HMPV=human metapneumovirus, PIV 1= parainfluenza virus type 1, HCoV 229/NL63=human coronavirus 229E/NL63, PIV 3= parainfluenza virus type 3, PIV 4= parainfluenza virus type 4)

Slika 4. *Učestalost detektiranih virusa u uzorcima s pozitivnim multipleks PCR testom na respiratorne viruse (HRV = humani rinovirus A/B/C, RSV A/B = respiratorni sincicijski virus A i B, AdV = adenovirus, HCoV OC43 = humani koronavirus OC43, Flu A/B = virus influence A i B, HBoV = humani bokavirus 1/2/3/4, HEV = humani enterovirus, HMPV = humani metapneumovirus, PIV 1 = virus parainfluence tip 1, HCoV 229/NL63 = humani koronavirus 229E/NL63, PIV 3 = virus parainfluence tip 3, PIV 4 = virus parainfluence tip 4)*

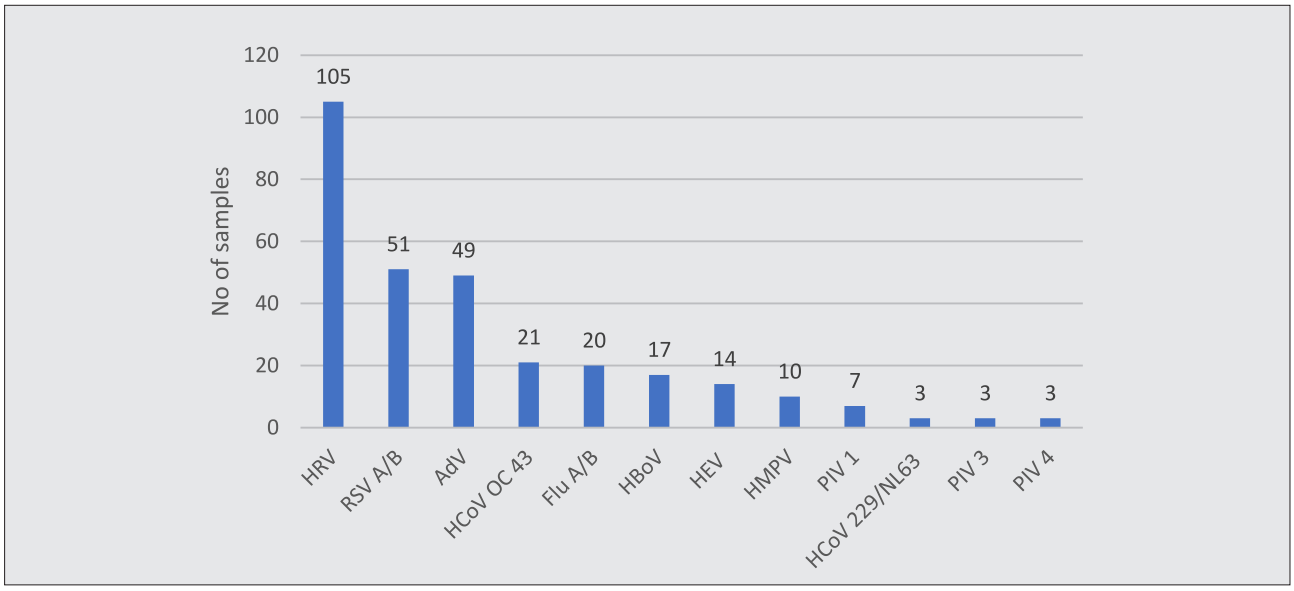


Figure 5. Age group distribution of children with detected human bocavirus (HBoV)

Slika 5. *Raspodjela po dobi ispitanika s detektiranim humanim bokavirusom (HBoV)*

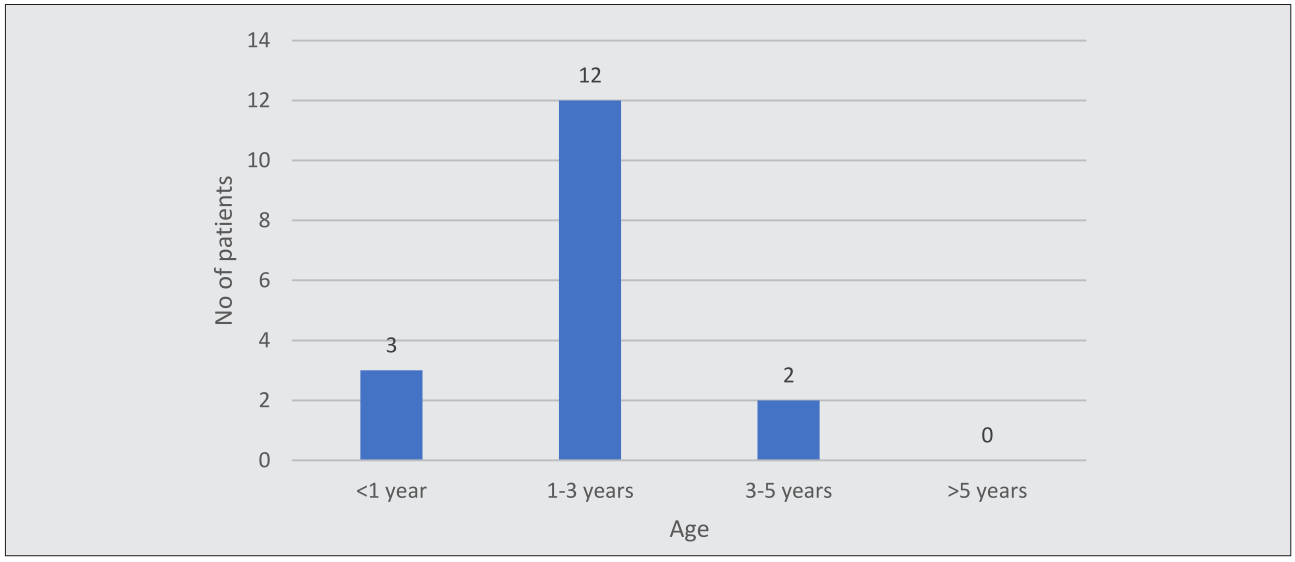


Figure 6. Localization of acute respiratory infection in patients with detected human bocavirus (HBoV) (URTI= upper respiratory tract infection, LRTI= lower respiratory tract infection)

Slika 6. *Lokalizacija akutne respiratorne infekcije kod pacijenata s detektiranim humanim bokavirusom (HBoV) (IGDS= infekcija gornjeg dišnog sustava, IDDS= infekcija donjeg dišnog sustava)*

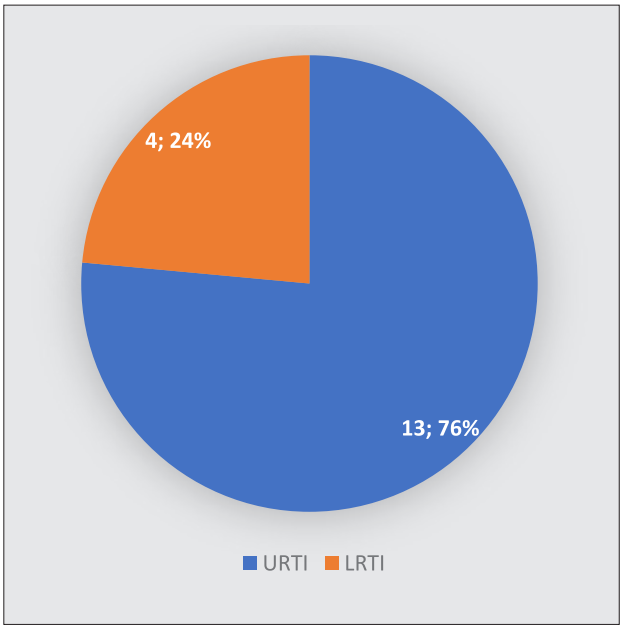


Figure 7. Monoinfection and coinfection rate of human bocavirus (HBoV) and HBoV with other respiratory viruses, respectively

Slika 7. *Učestalost monoinfekcije s humanim bokavirusom (HBoV) i koinfekcije HBoV s drugim virusima*

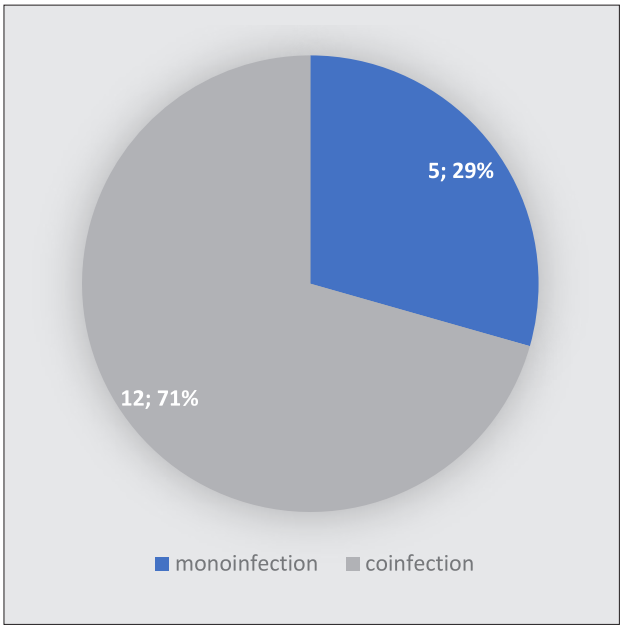


Figure 8. Number of viruses detected in coinfections with human bocavirus (HBoV)

Slika 8. *Broj detektiranih virusa u koinfekciji s humanim bokavirusom (HBoV)*

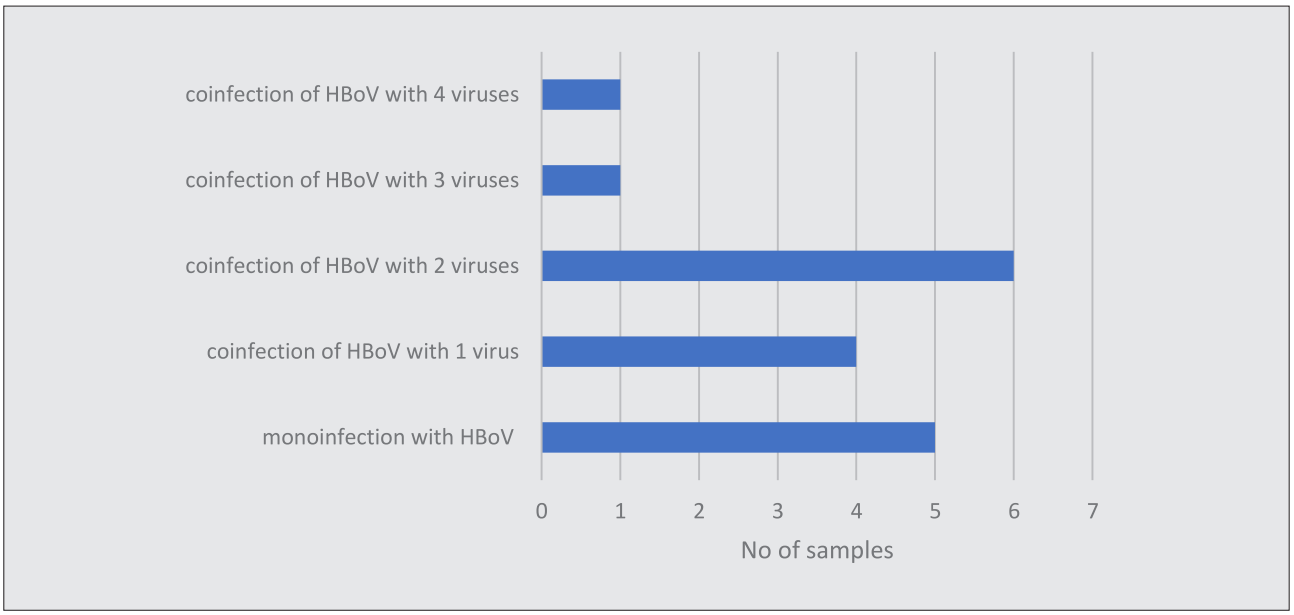
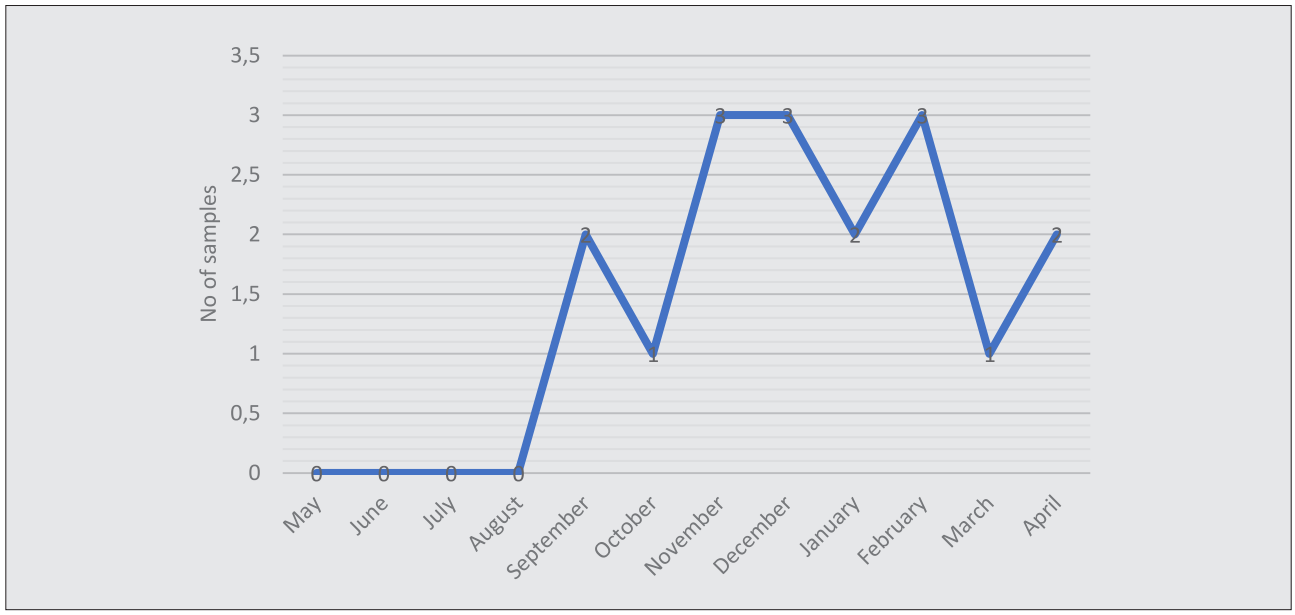


Figure 9. Temporal distribution of samples positive for human bocavirus (HBoV) in period of May 1st 2017- April 30th 2018 (N=17)

Slika 9. *Raspodjela uzoraka pozitivnih na humani bokavirus (HBoV) tijekom perioda od 1. svibnja 2017. do 30. travnja 2018. (N=17)*



Discussion

Since its discovery in 2005, many different studies have reported prevalence of HBoV in acute respiratory infections, ranging from 1.0% to 56.8%, depending on the country and population under investigation [2,3]. There are scarce data on HBoV prevalence in respiratory tract infection for Croatia. The first report of HBoV detection in Croatia revealed its frequency detection rate of 10.5% in respiratory samples of children with ARI but on small sample size (n=72) [27]. The most recent data from retrospective study that searched for HBoV in respiratory samples of 295 children with lower respiratory tract disease showed prevalence of 23.1% [28]. This report presents results from first year of 4-year prospective study revealing HBoV prevalence of 6.2% in respiratory samples of children with symptoms and signs of ARI of both, URTI and LRTI, which is lower than prevalence found in previously mentioned studies. However, all studies indicate that in a significant proportion of respiratory samples of Croatian children with ARI, HBoV was identified. The rate of co-infection/co-detection of HBoV with other respiratory viruses also varies in different studies worldwide, and ranges from 8.3% to 100%. [2,4]. In this study, HBoV was co-detected with other respiratory viruses in 70% of cases, which is highly consistent with continuous findings of high co-detection rate (87.5% and 51%) in other Croatian studies [27,28]. It is also in line with studies from Italy who showed average prevalence of 7.4% and average co-detection rate of 62.8% [4]. The highest prevalence averages in Europe have been reported for Hungary (29.8%) with co-detection with other respiratory viruses of 53.6 %. [4]. Prevalence studies from Germany found HBoV in 10.1 % of respiratory samples and average co-infection of 38.7% [4].

Although Koch's postulates cannot be completely fulfilled for HBoV, due to the lack of adequate animal and cell culture models, there is substantial evidence suggesting that HBoV1 is indeed an etiological pathogen of respiratory tract disease, rather than an innocent bystander, especially in children under five years of age [2]. Common respiratory clinical syndrome of upper and lower respiratory tract localization (i.e. otitis media acuta and bronchiolitis, respectively) are strongly associated with HBoV detection in young children [17, 29]. Some studies found that HBoV is, after rhinoviruses, RSV A/B and adenoviruses, most commonly associated virus in community-acquired viral pneumonia in childhood [30-32]. Moreover, newer studies suggest that severe HBoV infection relates to an increased risk of some chronic diseases (i.e. HBoV bronchiolitis in infancy is also strongly associated with recurrent wheezing and exacerbation of asthma) [33]. However, the unambiguous interpretation of the HBoV detection complicates the large proportion of its co-detection with other viruses as well as its finding in asymptomatic persons. The long persistence and long shedding of HBoV 1 in the nasopharynx, up to 50 days, could explain the high rate of co-detection with common respiratory microorganisms, but still complicates the interpretation of positive DNA amplification test results [34,35]. According to some authors, it seems that sole PCR positivity in the airways is not fully reliable diagnostic marker of primary human bocavirus infection. Detection of anti-HBoV 1 antibodies or viral DNA in serum or of HBoV 1 spliced mRNA, high copies of viral DNA or antigen in airway samples is a more reliable tool to detect primary infection [36-38].

As HBoV 1 serological assays are not yet commercially available, and due to high viral loads correlate with acute infections, fewer coinfections and increased disease severity, some authors advocate use of quantitative PCR for diagnosis of HBoV acute infection and cut off value of $>10^4$ HBoV 1 genomes/mL of nasopharyngeal secretion [10,39].

In this study, 12 of 17 (70. 6%) children with detected HBoV were between one and three years of age; they were older than RSV infected children, but younger than influenza infected children. This finding is also consistent with the results of previous Croatian study [28], who also showed that detection rates of HBoV infection increased with age, while RSV infection rates decreased with age. Few seroprevalence studies have revealed that protective maternal antibodies are present in infants younger than two months of age and after that seropositivity decreases with lowest prevalence in the 6- to 12-month age group [34,35], which may explain why most symptomatic infections occur between one and three years of age. HBoV 1 is detectable less frequently in other age groups including adults. The reason for that is probably because reinfection occurs throughout life and the high prevalence of antibodies remains into adulthood. Although it is generally accepted that reinfections in adults are mostly mild or asymptomatic, some studies conducted on tissue samples have discovered HBoV as a single viral agent in 17% mucosal samples from adults with chronic sinusitis, leaving possibility that HboV also plays pathogenic role in adults [40].

Similar as in other studies [39], HBoV was detected throughout whole year except summer months, predominantly during cold periods of year and peak low temperatures in winter months. However, to find out more about seasonality of this virus in Croatia, because of small sample number and one-year period of research, further research is needed.

In conclusion, although often detected in combination with other respiratory viruses, HBoV is one of the possible causes of ARI in children. Detection of HBoV in the respiratory secretion need to be included in standard respiratory viruses' panel for routine laboratory diagnosis of ARI. Continuous HBoV detection is required in order to complete our findings on clinical significance and epidemiology of this infection.

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Conflict of interest

Nothing to declare.

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