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ORIGINAL ARTICLE

# THE FREQUENCY OF RESPIRATORY VIRUSES IN LOWER RESPIRATORY TRACT INFECTIONS IN CHILDREN

# ZASTUPLJENOST RESPIRATORNIH VIRUSA U INFEKCIJAMA DONJIH PARTIJA RESPIRATORNOG TRAKTA U DEČJEM UZRASTU

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## Abstract

**Introduction:** Acute viral infections of the lower respiratory tract are of great importance for mortality in children worldwide. The most common causative agents include human respiratory syncytial virus (HRSV), human metapneumovirus, parainfluenza virus, influenza virus and adenoviruses. The application of molecular methods has significantly contributed to the identification and determination of the frequency of respiratory viruses in these infections.
**Aim:** The aim of this study was to determine the frequency of different viruses that cause lower respiratory tract infections in the pediatric population using panel PCR test from bronchoalveolar lavage samples.
**Material and methods:** Eighteen samples of bronchoalveolar lavage of children younger than 5 years with a diagnosis of pneumonia and antigenic confirmation of the presence of HRSV were analyzed. Virus detection was performed by real-time chain polymerization using the commercial FTlyo™ Respiratory Pathogens 21 kit (Fast Track Diagnostics, Luxembourg) to simultaneously detect the genomes of 18 different viruses and 1 bacterium.
**Results:** Respiratory viruses were detected in all 18 samples, with HRSV identified in all samples, human bocavirus (HBoV) in 7 and human rhinovirus (HRV) in one sample. Co-infections were detected in 38.9% of samples. The HRSV and HBoV co-infection was proven in 33.33% of samples, while the presence of all three viruses in only one sample.
**Conclusion:** The results of this study show the presence of HRSV, HBoV and HRV in bronchoalveolar lavage using multiplex RT-PCR method. Both HRSV and HBoV were the most common viral coinfections. Additional research is needed to more accurately estimate the frequency and distribution of respiratory viruses in children.

**Keywords:**
pediatrics,
acute respiratory infection,
respiratory viruses,
pneumonia,
multiplex RT-PCR



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## Sažetak

**Uvod:** Akutne virusne infekcije donjih partija respiratornog trakta od velikog su značaja za mortalitet dece širom sveta. U grupu najčešćih izazivača spadaju humani respiratorni sincicijalni virus (HRSV), humani metapneumovirus, virus parainfluence, virus influence i adenovirusi. Primena molekularnih metoda je značajno doprinela identifikaciji i utvrđivanju učestalosti respiratornih virusa u ovim infekcijama.

**Cilj:** Cilj ovog istraživanja je otkrivanje zastupljenosti različitih virusa koji izazivaju infekcije donjeg respiratornog trakta kod pedijatrijske populacije pomoću panel testova polimerazne lančane reakcije (PCR) iz uzorka bronhoalveolarnog lavata.

**Materijal i metode:** Analizirano je 18 uzoraka bronhoalveolarnog lavata dece mlađe od 5 godina sa dijagnozom pneumonije i antigenskom potvrdom prisustva HRSV. Detekcija virusa je vršena primenom komercijalnog testa *FTlyo™ Respiratory Pathogens 21 kit (Fast Track Diagnostics*, Luksemburg) za istovremeno dokazivanje genoma 18 različitih virusa i 1 bakterije.

**Rezultati:** Prisustvo respiratornih virusa je dokazano kod svih 18 uzoraka. Humani respiratorni sincicijalni virus je dokazan u svim uzorcima, humani bokavirus (HBoV) dokazan je u 7, a humani rinovirus (HRV) u jednom uzorku. Koinfekcije su povrđene kod 38,9% uzoraka. Koinfekcija HRSV i HBoV je dokazana u 33,33% slučajeva. Prisustvo sva tri virusa je utvrđeno u samo jednom uzorku.

**Zaključak:** Rezultati ove studije pokazuju prisustvo HRSV, HBoV i HRV u bronhoalveolarnom lavatu primenom multipleks RT-PCR metode. Najčešća virusna koinfekcija je bila HRSV i HBoV. Za precizniju procenu zastupljenosti i distribucije respiratornih virusa kod dece potrebna su dodatna istraživanja.



**Ključne reči:**
pedijatrija,
akutna respiratorna infekcija,
respiratorni virusi,
pneumonija,
multipleks RT-PCR


## Introduction

Acute respiratory infections are among the most common infections worldwide. The World Health Organization lists respiratory infections as the leading cause of infectious diseases. Infections of the lower respiratory tract are troublesome, especially in children under 5 years of age (1). Pneumonia and acute viral bronchiolitis are the main causes of hospitalization in regions with a high socioeconomic standard and are one of the leading causes of death in children under 5 years of age in developing countries (2).

Viruses in the lower respiratory tract affect the deeper structures below the larynx, which include the trachea, bronchus and bronchoalveoli. This manifests as bronchiolitis, bronchitis and acute pneumonia. Children, older than 65 years, patients with other respiratory diseases and those with a suppressed immune system have an increased risk for complications due to respiratory infection. Decreased or increased mucociliary function can lead to decreased clearance of the viral pathogen and thus increase the risk of infection. Children are at risk of complications due to an insufficiently developed immune system and physiological differences in the respiratory tract between children and adults which make children more susceptible to viral infections. The immune response to respiratory viral infection can be enhanced by protective passive antibodies transferred in utero or through breastfeeding (1). Factors that increase the chance of a more severe clinical presentation are prematurity, low birth weight, chronic cardiopulmonary diseases, congenital or acquired immunodeficiency disorders, malnutrition, a large number of children in the household, air pollution, passive smoking and children who are not breastfed (3).

The leading causes of acute respiratory infections in the pediatric population are human respiratory syncytial virus (HRSV), adenovirus and rhinovirus, while human metapneumovirus and human coronavirus are less frequent (1). In hospital conditions, the most common cause is adenovirus, followed by influenza viruses A and B and HRSV (4). Adenovirus is most prevalent in children aged 3 to 6 years, influenza A and B are more common in children older than 6 years, while HRSV is most common in the population under one year of age. Viral infections are more prevalent during the winter period (5).

Infections of the respiratory tract with different viruses may be presented with similar signs and symptoms, which is why it is difficult to determine the cause based on the clinical presentation. Rapid diagnosis shortens the duration and cost of hospital treatment, reduces the number of additional laboratory tests and reduces the use of antibiotics. Various antigenic tests have been the basis of the diagnosis of viral respiratory infections for a long time. Advances in the fields of molecular medicine have led to the increasing use of these techniques in laboratory diagnosis. Today, tests based on nucleic acid amplification such as polymerase chain reaction (PCR) are frequently used in routine diagnostics (6). Multiplex panel tests allow the detection of several different viruses and the identification of possible co-infections in one sample (7).

The aim of this study was to determine the frequency of different viruses that cause lower respiratory tract infections in the pediatric population using a panel PCR test from a bronchoalveolar lavage sample.

## Material and methods

### Clinical samples

The archival eighteen samples of bronchoalveolar lavage from children under 5 years of age with a diagnosis of pneumonia from the Institute for Health Care of Mothers and Children of Serbia "Dr. Vukan Čupić" from October to December 2019 were used in this study. In all specimens, the presence of human respiratory syncytial virus was previously proven by an antigen test (RSV Rapid Antigen test, BD, USA). After the HRSV antigen confirmation, the bronchoalveolar lavages were transported to the virology laboratory of the Institute of Microbiology and Immunology, Faculty of Medicine University of Belgrade and stored at -80°C until the analysis.

### Methods

The detection of viruses in the samples was performed using a molecular technique for simultaneous detection of the different viral genomes in real time (Multiplex Real-time PCR). The test used in this study detects influenza A virus (IAV), influenza A (H1N1) virus (IAV (H1N1) swl), influenza B virus (IBV), human coronavirus (HCoV 229E, HCoV NL63, HCoV HKU1, HCoV OC43) , human parainfluenza virus (HPIV-1, HPIV-2, HPIV-3 and HPIV-4), human metapneumovirus (HMPV A and HMPV B), human rhinovirus (HRV), human respiratory syncytial virus (HRSV A and HRSV B), human adenovirus (HAdV), enterovirus (EV), human parechovirus (HPeV), human bocavirus (HBoV) and Mycoplasma pneumoniae.

The procedure consisted of the following: 1) extraction of nucleic acids, 2) Real-time PCR (RT-PCR) and 3) the interpretation of the results.

### Extraction of DNA and RNA

The DNA extraction was performed using the PureLink™ Genomic DNA Mini Kit (Invitrogen, USA), while the QIAamp Viral RNA Mini Kit (QIAGEN, Germany) was used for RNA extraction. Nucleic acid extraction was performed according to the manufacturer's protocols for bronchoalveolar lavage samples. The obtained DNA and RNA extracts were used for amplification.

### Real-time PCR amplification

The FTlyo™ Respiratory Pathogens 21 kit (Fast Track Diagnostics, Luxembourg) was used, which simultaneously detects 18 viruses and 1 bacteria according to the manufacturer's instructions. The test consists of 5 mixes that detect different viruses, M.pneumoniae and an internal control. The positive and negative controls were used for each mix (**figure 1**).

### Interpretation of results

Amplification results were interpreted based on the reading of the fluorescence intensity of the amplification curve, i.e. threshold cycle Ct. Fluorescence values of different colors were read in all mixes, and the result was determined based on the test instructions (**figure 2**).

Due to the small sample size, the frequency and percentage were used in order to compare the results with the literature finding.

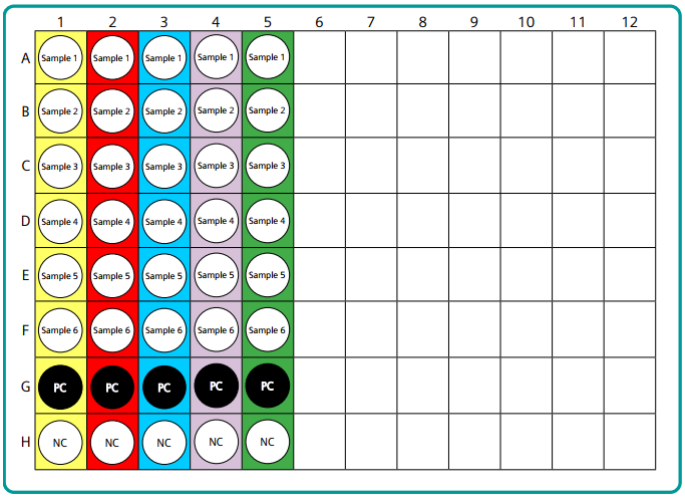


**Figure 1.** Layout of the instructions for the layout of the mixes when performing the test. Yellow = FluoRhino master mix (A1-H1), Red = COR master mix (A2-H2), Blue = ParaEAV master mix (A3-H3), Purple = BoMpPf1 master mix (A4-H4), Green = RsEPA master mix ( A5-H5), PC = positive control (G1-G5), NC = negative control (H1-H5).

## Results

The presence of respiratory viruses was proven in all 18 samples. In total, the presence of 3 viruses was proven, namely human respiratory syncytial virus, human bocavirus and human rhinovirus. Human respiratory syncytial virus was detected in all samples (100%), human bocavirus was detected in 7 out of 18 samples (38.89%), human rhinovirus was detected in only one sample (5.56%). Of the tested samples, 11 (61.1%) were positive for only one virus (human respiratory syncytial virus). Co-infection of human respiratory syncytial virus and human bocavirus was proven in 6 cases (33.33%). While the presence of all three viruses was proven only in one sample (**figure 3 and 4**). The presence of internal control was proven in all samples.

Out of 18 samples, 11 belong to newborns who are not older than one month, the other 7 samples belong to children younger than 5 years old. Patient characteristics differ concerning gender, age and sample collection time (**table 1**).

Statistical analysis was not performed due to the small number of tested samples.

## Discussion

Acute pneumonia acquired outside the hospital is

Master mix

| Pathogens | FluRhino | COR | ParaEAV | BoMpPf1 | RSEPA | Dye | Wavelength (nm) |
|---|---|---|---|---|---|---|---|
| | IAV | HcoV 229E | HPIV-3 | HPIV-1 | HRSV A i B | Green (FAM) | 520 |
| | HRV | HcoV NL63 | HPIV-2 | HMPV A i B | HPeV | Yellow (JOE) | 550 |
| | IBV | HcoV HKU1 | HPIV-4 | HBoV | EV | Orange (ROX) | 610 |
| | IAV (H1N1) swl | HcoV OC43 | IC (EAV) | *M.pneumoniae* | HadV | Red (Cy5) | 670 |

**Figure 2.** Method of reading the results based on the presence of fluorescence amplification curves of different colors in the mixes. Test tags: influenza A virus (IAV), influenza A (H1N1) virus (IAV (H1N1) swl), human coronaviruses (HcoV 229E, HcoV NL63, HcoV HKU1, HcoV OC43), human parainfluenza viruses (HPIV-3, HPIV -1, HPIV-2, HPIV-4), human respiratory syncytial virus (HRSV A and B), human rhinovirus (HRV), human metapneumovirus (HMPV A and B), human parechovirus (HPeV), influenza virus B (IBV) , human bocavirus (HBoV), enterovirus (EV), internal control (IC (EAV)), Mycoplasma pneumoniae (M.pneumoniae), human adenovirus (HadV).

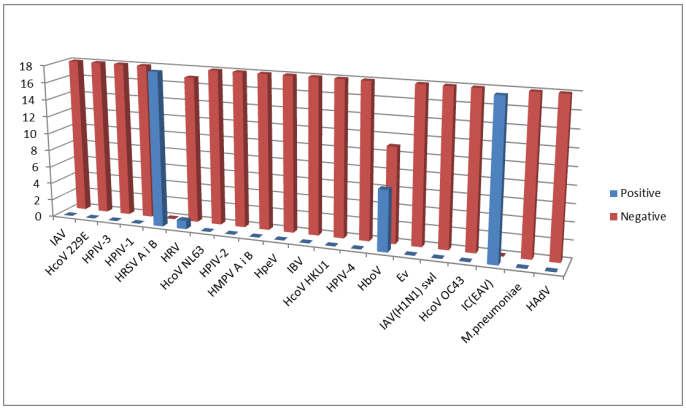


**Figure 3.** Presentation of the frequency of respiratory viruses in the analyzed samples.

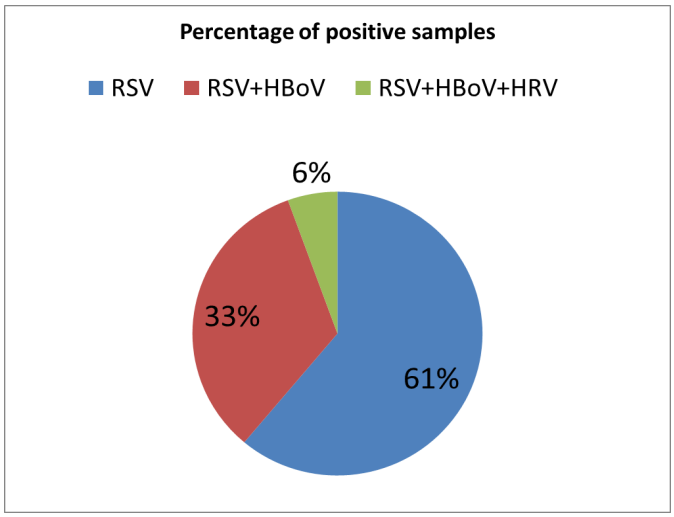


**Figure 4.** Percentage of virus in positive samples.

defined as an acute infection of the lower parts of the respiratory tract (lasting less than 14 days) acquired in the community, which manifests in the form of cough, difficulty breathing, tachypnea, and retraction of the soft tissues of the chest during inhalation. Bronchiolitis is a viral infection of the lower parts of the respiratory tract that predominantly occurs in children under 2 years of age, especially in newborns (2).

Viral infections begin in the upper parts of the respiratory tract and spread to the lower parts in a few days. Inflammation of the bronchiolar epithelium, edema of the submucosa and adventitia occurs. Trapped air distal to the airway obstruction together with atelectasis and an imbalance in pulmonary ventilation and perfusion leads to hypoxemia. These mechanisms are known in HRSV bronchiolitis, but are considered to be valid for other viruses that cause a similar pathological picture (2).

Community-acquired pneumonia in the pediatric population is a leading cause of respiratory morbidity and mortality worldwide (8). Infections of the lower parts of the respiratory tract are most often of viral origin. The definition of acute lower respiratory tract infection very often includes acute pneumonia and acute bronchiolitis in children. Acute pneumonia and acute viral bronchiolitis are common reasons for hospitalization in regions with high socioeconomic standards, while pneumonia is the leading cause of death in children under 5 years of age in developing countries. Acute lower respiratory tract infections are the reason for hospitalization in 30 - 40% of cases, while in developing countries they account for 15 - 28% of the cause of death (2). The prevalence of respiratory viruses

**Table 1.** Patient characteristics concerning gender, age and sample collection time.

| Sample | Gender | Time of sampling | Age | HRSV | HBoV | HRV |
|---|---|---|---|---|---|---|
| 1 | M | December | Newborn | + | - | - |
| 2 | F | December | Newborn | + | - | - |
| 3 | M | December | Newborn | + | + | - |
| 4 | F | December | < 5 godina | + | + | - |
| 5 | M | December | Newborn | + | + | - |
| 6 | F | December | < 5 years | + | - | - |
| 7 | M | December | < 5 years | + | + | - |
| 8 | F | December | Newborn | + | + | - |
| 9 | F | December | < 5 years | + | + | + |
| 10 | F | December | < 5 years | + | - | - |
| 11 | M | December | Newborn | + | + | - |
| 12 | F | November | < 5 years | + | - | - |
| 13 | F | | Newborn | + | - | - |
| 14 | F | December | Newborn | + | - | - |
| 15 | M | December | Newborn | + | - | - |
| 16 | M | December | Newborn | + | - | - |
| 17 | M | December | < 5 years | + | - | - |
| 18 | F | October | Newborn | + | - | - |

F = Females; M = Males; - negative result; + positive result

is drastically higher in the population group younger than 3 years compared to older children (8). The incidence of pneumonia acquired outside the hospital is from 36 to 40 cases per 1000 children under 5 years of age on an annual basis. In children aged 5 to 14, the incidence is 11 to 16 per 1000 patients. It is estimated that the number of pneumonia cases annually in Europe is 2.5 million (9).

In Serbia, there are a limited number of studies that monitor the frequency of respiratory viruses within the pediatric population. In this research, bronchoalveolar lavage samples from 18 patients were examined using the molecular multiplex RT-PCR method. Samples that were positive for HRSV by antigen test were analyzed, which was also confirmed by our molecular method. Multiplex panel test used in this study offers the possibility of detecting 19 pathogens, 3 of which were detected in our samples, namely HRSV, HRV and HBoV. Human respiratory syncytial virus is the most common cause of acute viral pneumonia, especially in the first year of life (2). Bronchiolitis caused by HRSV is most often seen in infants where the frequency of this virus is 2 to 3 times higher than in older children. The number of new episodes of acute lower respiratory tract infection caused by HRSV was estimated to be 33.8 million worldwide in 2005 in children under 5 years of age. About 66,000 to 199,000 children under 5 years of age died from HRSV infection in 2005, with 99% of deaths occurring in developing countries (2). The HRSV is predominantly detected in autumn and winter (8). The results of this study are also in line with this since positive samples from November and December were analyzed. Studies estimate a prevalence of HBoV of 13% in the population under 2 years of age (10). The largest number of children infected with HBoV are younger than 24 months (11). The frequency of HBoV in children with bronchiolitis is thought to vary from 1.8% to 37% in different countries (10). As is the case in one study where HBoV is the second most common virus (19.5%), just behind HRSV (43.4%) (12). Geographical location is considered a determining factor in the prevalence of HBoV. Studies also indicate that HBoV is more often seen in co-infections with other viruses than alone, but it is still unknown whether this affects the severity of the clinical picture (11). The diagnosis of HBoV infection is most often made in winter and spring (10).

In a study of 2525 cases of children younger than 14 years of age, 599 patients had an infection caused by HRSV, while 326 had coinfections (13). The HRSV has frequent coinfections with other respiratory viruses that circulate in the same seasonal patterns such as influenza, human rhinovirus, human metapneumovirus, and human bocavirus. The most common dual infection was between HRSV and HRV (120 cases), followed by coinfection between HRSV and HBoV (60 cases). Other HRSV dual infections were with HMPV and influenza (13). Numerous studies have confirmed that respiratory viruses, especially HRSV and HRV, can predispose to recurrent wheezing in the early years of life and possible asthma (2). In this study, coinfections were proven in 38.9% of cases, primarily coinfections with two viruses. The highest frequency of coinfections was between HRSV and HBoV (33.33%). HRV was detected in only one sample together with HRSV and HBoV (5.56%). These results can be explained by the small number of tested samples that come from only one clinic in Belgrade, but also due to the possible different geographical spread of the virus in our population compared to other countries.

There is a need for fast, sensitive and accurate

diagnosis of respiratory infections in children, the elderly and the immunocompromised. Detection methods based on nucleic acid amplification such as PCR and RT-PCR are increasingly used to detect viruses in infectious respiratory diseases. Multiplex RT-PCR is used worldwide for the simultaneous detection of different viruses due to its high specificity and sensitivity. Fast and early virological diagnosis is important, not only for the decision in therapy but also plays an important role in the prevention of nosocomial infections and the monitoring of new epidemics. Using standard diagnostic methods such as virus isolation from cell culture or antigen tests requires more time than molecular methods. Multiplex assays can also be used in epidemiological studies to monitor the frequency of respiratory viruses (14). Today, the costs of these multiplex tests are still high, but there are certain populations where their use would be appropriate, such as pediatric patients with other comorbidities (1).

## Conclusion

The results of this study show the presence of different respiratory viruses in the pediatric population in the bronchoalveolar lavage sample using the multiplex RT-PCR method. Three viruses were proven, namely HRSV (100%), HBoV (38.89%) and HRV (5.56%). Viral co-infections were detected in 38.89% of cases where the most common was between HRSV and HBoV. For a more detailed assessment of the frequency and distribution of respiratory viruses in children, additional research is needed on a larger sample size from several health institutions in Serbia.

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