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CLINICAL MICROBIOLOGY - RESEARCH PAPER

Viral pathogens associated with acute lower respiratory tract infections in children younger than 5 years of age in Bulgaria

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Abstract
Acute lower respiratory infections (ALRIs) are a leading cause of morbidity and hospital admissions in children. This study aimed to determine the viral etiology of these infections in children aged < 5 years during three successive epidemic seasons in Bulgaria. Nasopharyngeal and throat specimens were collected from children with bronchiolitis and pneumonia during the 2015/2016, 2016/2017, and 2017/2018 seasons. The viral etiology was determined by individual real-time PCR assays against 11 respiratory viruses. Of the 515 children examined, 402 (78.1%) were positive for at least one virus. Co-infections with two and three viruses were found in 64 (15.9%) of the infected children. Respiratory syncytial virus (RSV) was the predominant pathogen (37.5%), followed by rhinoviruses (13.8%), metapneumovirus (9.1%), adenoviruses (7%), bocaviruses (7%), influenza A(H1N1)pdm09 (4.9%), A(H3N2) (4.3%), type B (4.1%), and parainfluenza viruses 1/2/3 (2.9%). RSV-B were more prevalent than RSV-A during the three seasons. At least one respiratory virus was identified in 82.6% and 70.1% of the children with bronchiolitis and pneumonia, respectively. Respiratory viruses, especially RSV, are principal pathogens of ALRIs in children aged < 5 years. Diagnostic testing for respiratory viruses using molecular methods may lead to the reduced use of antibiotics and may assist in measures to control infection.

Keywords Acute lower respiratory infections · Bronchiolitis · Pneumonia · Viral infection

Introduction

Acute lower respiratory infections (ALRIs) are a major cause of morbidity and the leading infectious cause of death in children younger than 5 years on a global scale. They represent an important health and social problem due to the associated large number of doctors' office visits, hospitalizations, work hours lost by parents, and enormous medical and socio-economic costs. A systematic review estimated that, in 2010, 11.9 million episodes of severe and 3 million episodes of very severe ALRIs resulted in hospital admissions of young children worldwide [1]. Based on pathophysiologic processes, ALRIs can be divided into bronchiolitis and pneumonia. Bronchiolitis affects the smallest branches of the bronchial tree in children < 2 years of age and most often has viral etiology, whereas pneumonia can be caused by bacteria, viruses, or other pathogens [2]. A wide range of different viruses can cause ALRI of varying severity [3]. Strong similarities in the clinical manifestations of different viral infections make diagnosis based on clinical parameters unreliable. The use of sensitive nucleic acid–based molecular techniques in current laboratory practice allows rapid and accurate etiological diagnosis, which can help in clinical decision making and may assist in measures to prevent nosocomial transmission.

Influenza viruses cause annual epidemics, during which 5–10% of adults and 20–30% of children are affected [4]. Children aged under 5 years, particularly children aged < 2 years, are a major risk group for severe influenza and complications. It was estimated that, in 2008, 20 million cases of

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influenza-associated ALRI and 1 million cases of influenza-associated severe ALRI occurred worldwide in children younger than 5 years [5]. Respiratory syncytial virus (RSV) is a common cause of ALRI in infants and young children and is the most frequently identified pathogen in cases of bronchiolitis. In 2015, this virus caused 33.1 million episodes of ALRI worldwide and resulted in approximately 3.2 million hospital admissions and 59,600 in-hospital deaths in children younger than 5 years [6]. Human metapneumovirus (hMPV) is closely related to RSV in its structural, epidemiologic, and clinical characteristics, with disease severity similar or less than RSV [7, 8]. Human parainfluenza virus (PIV) types 1 and 2 are major etiologic agents of laryngotracheobronchitis (croup); PIV-3 and to a lesser extent PIV-1 are frequently associated with bronchiolitis and pneumonia in infants and young children [9]. The clinical spectrum of human rhinovirus (RV) infections ranges from a mild upper respiratory tract illness to severe pneumonia, and a significant proportion of the infections (~1/3) are asymptomatic. Rhinoviral ALRIs in early childhood, such as RSV infections, are associated with recurrent wheezing and asthma later in life [10]. Human adenoviruses (AdV) cause a wide range of respiratory diseases of variable severity and are a particular problem in immunocompromised children [11]. The human bocaviruses (BoV) are characterized by high levels of co-infections [12].

The aim of this study was to determine the incidence of 11 of the most common respiratory viruses, namely influenza viruses, RSV, hMPV, PIV 1/2/3, RV, AdV, and BoV, among children aged 0 to 5 years with ALRI and to characterize the epidemiologic features of infections during three successive epidemic seasons.

Materials and methods

Study population and specimen collection

This study started in October 2015 and covered three consecutive winter seasons (2015/2016, 2016/2017, and 2017/2018). Patients were recruited from October to May inclusively. In 2017, surveys were made all year round. The study population comprised children aged 0–5 years from different regions of Bulgaria treated for ALRI (bronchiolitis or pneumonia) in primary care settings or hospitals. Written informed consent from parent/guardian was obtained before enrollment. Children were only once included in the study. The diagnosis of each patient was determined by their attending pediatric physician based on standard clinical criteria. Bronchiolitis was defined as a combination of clinical symptoms and signs occurring in children aged less than 2 years including a viral upper respiratory infection prodrome, progressing to cough, tachypnea, expiratory dyspnea, prolonged expiration, and wheezing from a distance. Pneumonia was diagnosed based on clinical manifestations and X-ray changes. Diagnostic tests that were performed included blood analyses—complete blood count with differential picture and C-reactive protein (CRP). Chest X-ray and bacterial cultures of respiratory specimens were performed only in children with clinical suspicion of bacterial complication. The patients with suspected viral etiology of illness (normal or slightly elevated white cell count and CRP) were selected. Children were excluded from the study if they had the following: (a) an underlying medical conditions increasing the risk of respiratory infections, including prematurity, bronchopulmonary dysplasia, cystic fibrosis, congenital heart disease, and neurological or metabolic disease such as diabetes mellitus, hemoglobinopathies, immunosuppression, malignances; (b) hospitalization for any cause within the preceding 30 days.

Both nasopharyngeal and throat specimens were prospectively collected from all enrolled children (2 swabs per child) with the help of commercial polyester swabs (Deltalab, Spain) during the visit to the doctor or within the first 24 h of admission. The specimens were collected within 7 days of the respiratory symptoms onset. After collection, specimens accompanied with demographic data and clinical diagnosis were promptly transported with ice packs to the National Laboratory "Influenza and ARD" for viral respiratory pathogen detection. If immediate delivery was not possible, the samples were stored in a refrigerator (4–8 °C) for no more than 48 h. In the laboratory, both swabs from each child were stirred into a single tube containing 2 ml of sterile phosphate buffered saline (PBS) and antibiotics. The specimens were processed immediately or stored at − 80 °C before testing.

Extraction of nucleic acids and real-time RT-PCR

Viral nucleic acids were extracted automatically from respiratory specimens using a commercial ExiPrep Dx Viral DNA/RNA kit (Bioneer, Korea) in accordance with the manufacturer's instructions. The detection and typing/subtyping of influenza viruses were carried out by a real-time RT-PCR method and the SuperScript III Platinum® One-Step qRT-PCR System (Invitrogen, Thermo Fisher Scientific, USA). Primers and probes were provided by the International Reagent Resource (IRR, USA). All samples were first tested for the presence of influenza A and B viruses. Those that were positive for influenza A were subsequently screened for A(H1N1)pdm09 and A(H3N2). The genetic lineage of detected influenza B viruses was also determined by real-time RT-PCR. Amplification was performed using a Chromo 4 thermal cycler (Bio-Rad Laboratories, Inc., USA) in accordance with the protocol of CDC-Atlanta [13]. The detection of RSV, hMPV, PIV 1/2/3, RV, AdV, and BoV was performed using singleplex real-time PCR assays and an AgPath-ID One-Step RT-PCR kit (Applied Biosystems, Thermo Fisher Scientific, USA). The primers, probes, and thermocycling conditions used in the study were identical to those previously described

[14, 15]. Positive and negative controls were included in each run. For influenza type A and type B viruses, positive controls were provided by IRR, USA; for other targets, AmpliRun DNA/RNA Amplification Controls (Vircell, Spain) were used. Clinical specimens were tested in a separate real-time RT-PCR reaction for the RNAase-P gene, which provided verification of RNA integrity and the absence of PCR inhibition [13]. Subgroup-specific primers and probes targeting the RSV F and N genes were used for multiplex real-time RT-PCR to distinguish RSV-A from RSV-B, respectively, as described previously [16].

Statistics

Age, gender, clinical features, and the incidence of each virus were compared using the chi square or Fisher's exact tests for categorical variables. p values < 0.05 were considered statistically significant.

Results

Patient characteristics

The study population consisted of 515 children aged < 5 years suffering from ALRI: 112, 191, and 193 in the first, second, and third seasons, respectively, and 19 outside the season in 2017. Only 6 (1.2%) of these children attended outpatient healthcare centers, 509 (98.8%) were hospitalized, and 26 were admitted to intensive care units (ICUs). The patients' ages ranged from 13 days to 59 months (mo) (mean age 21.29 ± 15.20 mo; median age 20 mo). Among the study subjects, 90 (17.5%) were < 6 mo old, 78 (15.1%) were 6–11 mo old, 117 (22.7%) were 12–23 mo old, and 223 (43.3%) were 24–59 mo old, while 7 (1.4%) were an unknown age. Most children (55.3%) were under 23 mo of age; 306 (59.4%) participants were boys, and 209 (40.6%) were girls.

Respiratory virus identification

Viral respiratory pathogens were identified in 402 (78.1%) of the 515 patients examined: 90 (80.4%), 158 (82.7%), and 139 (72%) in the 2015/16, 2016/17, and 2017/18 seasons, respectively, and 15 (78.9%) in the summer of 2017. Single infections were detected in 338 (65.6%) children; co-infections with two and three viruses were found in 58 (11.3%) and 6 (1.2%) children, respectively. In 68 (13.2%) of the examined patients, the following influenza viruses were identified: 25 (4.9%) A(H1N1)pdm09, 22 (4.3%) A(H3N2), and 21 (4.1%) type B (17 B/Yamagata and 4 B/Victoria). The non-influenza viruses RSV, RV, hMPV, AdV, BoV, and PIV1/2/3 were detected in 193 (37.5%), 71 (13.8%), 47 (9.1%), 36 (7%), 36 (7%), and 15 (2.9%) of the examined patients, respectively. The pathogen most frequently identified was RSV, accounting for 48% of the infections with positive viral detection. In the cases of mixed infections, AdV, BoV, and InfA(H3N2) were more common co-infecting viruses, with the combinations RSV/AdV and InfA(H3N2)/RSV being the most frequently identified (Fig. 1; Table 1).

Among the 193 RSV-positive patients, RSV subgroup A were identified in 57 (29.5%) and RSV subgroup B in 121 (62.7%) patients. RSV-B outnumbered RSV-A over the three seasons. Co-infection with both subgroups of RSV was detected in 9 (4.7%) children. For 6 specimens (3.1%), the subgroup could not be determined due to a low viral load.

Seasonal distribution of viral agents

The greatest number of respiratory viruses was detected in specimens obtained in February 2016 and 2017 and April 2018 (Fig. 2). The study did not involve BoV testing in the first surveillance period. Influenza virus circulation was observed from December to April during the first two seasons and from December to February during the third season. RSV was identified in specimens collected between January 2015 and April 2016, October 2016 and April 2017, and December 2017 and April 2018. Furthermore, RSV activity peaked in week 5 of 2016, week 7 of 2017, and week 11 of 2018. In 2017, RV and BoV infections were more prevalent in the fall and the spring, whereas hMPV, PIV, and AdV occurred predominantly in the winter-spring seasons.

Age and gender distribution

Viral pathogens were detected in all age groups, and the positive rate in each of the four age groups was 73.3%, 80.8%, 74.4% ,and 80.7% (Fig. 3). The proportion of influenza-positive children was larger in the 24–59-mo age group (18.8%) compared to 0–23 mo (8.8%) ($p < 0.05$). The median age of children with influenza A(H1N1)pdm09 (19 mo) and type B (19 mo) infection was slightly higher than that of children with influenza A(H3N2) (17 mo). RSV was the most frequently detected pathogen among children with ALRI in all age groups studied. The prevalence of RSV infection was highest in the youngest age group (< 6 mo, 50%), followed by 6–11 mo (38.5%). The mean age (± SD) of children with proven RSV infection was 21.05 ± 15.22 mo, and the median age was 20 mo. Of the RSV-positive children with ALRI, 58.5% were under 2 years of age: 61% were boys and 39% were girls ($p = 0.5773$). The detection rates of other respiratory viruses fluctuated among the different age groups, but the differences were not statistically significant. Co-infections were more frequent in the 12–23-mo group (25.3%).

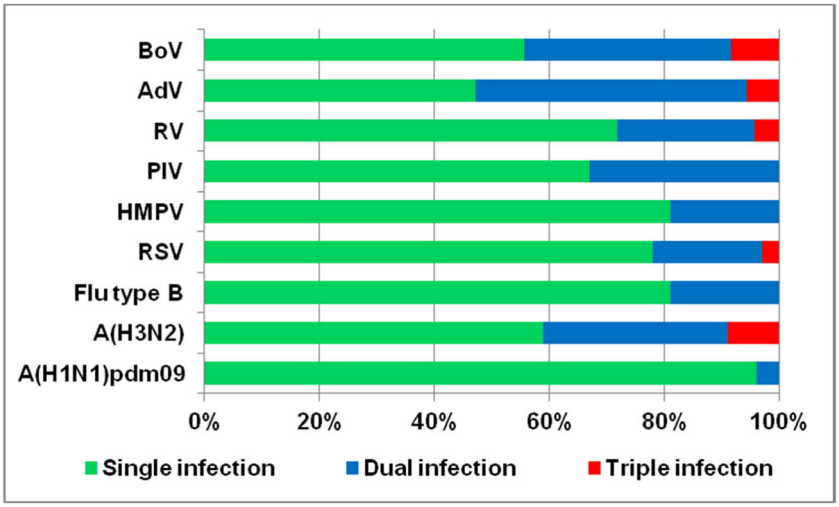

Fig. 1 Proportions of single infections and co-infections

Viral detection in children with different clinical diagnoses

Complications of the lower respiratory tract—bronchiolitis and pneumonia—were analyzed in 328 and 187 patients, respectively. Table 2 summarizes the baseline characteristics and the results of viral detection in the group of children with different clinical diagnoses.

Patients with different clinical diagnoses did not differ significantly in terms of age. Bronchiolitis and pneumonia cases occurred mainly during the winter season. Viral infections were detected in 271 (82.6%) patients with bronchiolitis and 131 (70.1%) with pneumonia. Among the patients with bronchiolitis, there were 42 cases of dual infections and 3 cases of triple infection; among the patients with pneumonia, there were 16 and 3 dual and triple infections, respectively. The proportion of detected influenza viruses among patients with bronchiolitis was 28 out of 328 (8.5%) and 40 out of 187 (21.4%) for pneumonia. Regarding non-influenza viruses, the proportions were 243/328 (74.1%) and 91/187 (48.7%), respectively. In the cases of pneumonia, influenza viruses were detected more commonly in children aged 24–59 mo as compared to those under 24 mo of age, whereas non-influenza viruses more often caused pneumonia in children under 24 mo of age ($p < 0.05$). In a 3-year-old child who died of pneumonia and acute respiratory distress syndrome (ARDS), influenza A(H1N1)pdm09 virus was detected. The most common virus associated with bronchiolitis was RSV ($p < 0.05$), accounting for 44.5% of the cases (115 cases as a single pathogen and 31 cases as part of co-infections), followed by RV (16.5%). Among the children suffering from pneumonia, RSV was also the most prevalent virus (25.1%)

Table 1 Number of respiratory viruses, detected as a single pathogen or co-pathogen, in children with ALRI*

	A(H1N1) pdm09	A(H3N2)	Inf typeB	RSV	hMPV	PIV1	PIV2	PIV3	RV	AdV	BoV	Detection rate (%)
A(H1N1)pdm09	*24*	0	0	1	0	0	0	0	0	0	0	4.9
A(H3N2)		*13*	0	6	0	0	0	0	0	1	0	4.3
Inf type B			*16*	3	0	0	0	0	1	1	0	4.1
RSV				*150*	1	0	0	1	5	8	5	37.5
hMPV					*38*	1	0	0	3	4	0	9.1
PIV1						*1*	0	0	1	0	1	0.8
PIV2							*1*	0	0	0	0	0.2
PIV3								*8*	0	0	1	1.9
RV									*51*	2	5	13.8
AdV										*17*	1	7.0
BoV											*20*	7.0
Co-pathogen in dual infection	1	7	5	37	9	3	–	2	17	17	13	
Co-pathogen in triple infection	–	2	–	6	–	–	–	–	3	2	3	

*Single pathogens are indicated in italics

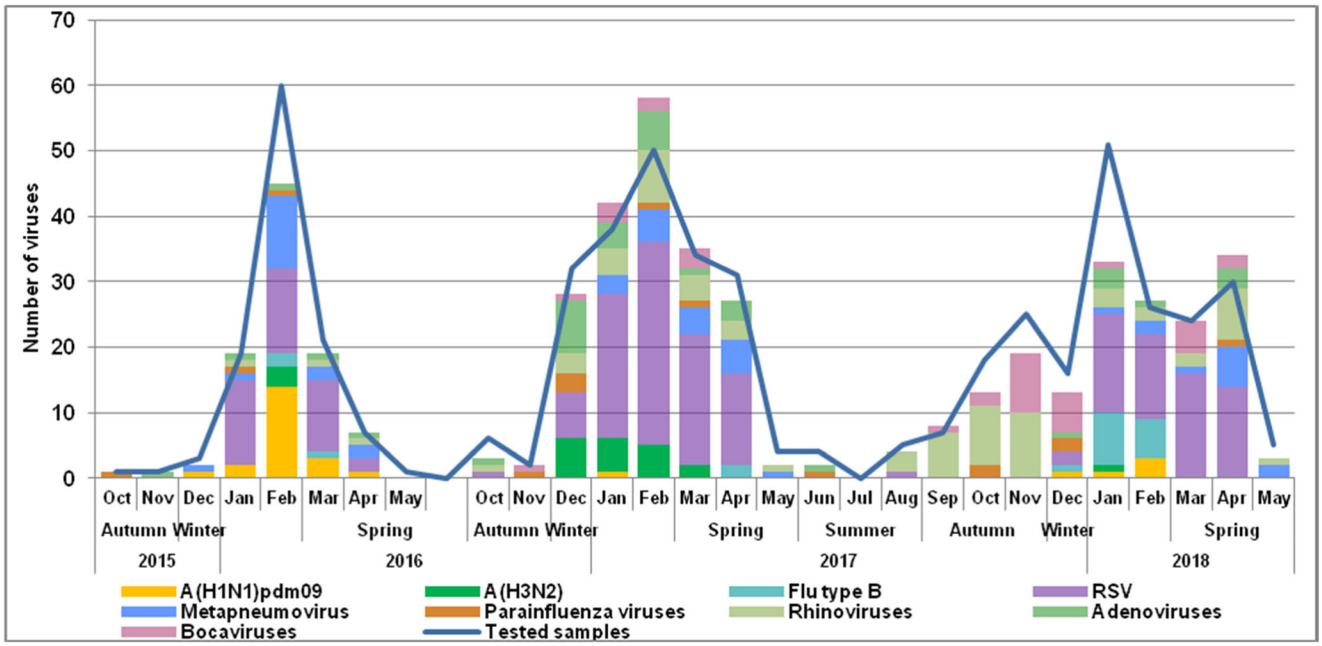

Fig. 2 Monthly distribution of respiratory virus detection among children with ALRI

($p < 0.05$), followed by AdV (11.2%) hMPV and RV (9.1%). In a 2-year-old child with pleuropneumonia and ARDS, RSV-A was detected. RSV and RV were more common pathogens of bronchiolitis than pneumonia, while influenza viruses and AdV were a more frequent cause of pneumonia compared to bronchiolitis ($p < 0.05$). Influenza A(H1N1)pdm09 and type B viruses, hMPV, and PIV were mostly detected as a single pathogen in cases of bronchiolitis and pneumonia, while AdV, BoV, RV, influenza A(H3N2), and RSV were frequently identified in mixed infections. In cases of bronchiolitis, AdV was more commonly found as a part of mixed infections than as a single pathogen. In 26 children treated in the ICU, 12 (46.2%) RSV, 1 (3.8%) hMPV, 2 (7.7%) PIV, 5 (19.2%) RV, and 5 (19.2%) BoV were detected.

Discussion

In this prospective study, we evaluated the incidence of the 11 most common respiratory viruses in 0- to 5-year-old children with ALRI in Bulgaria. During the study period, viral infections were detected in 78.1% of the cases of ALRI, with the lowest positive rate in the third season ($p < 0.05$). In the PCR-based surveys conducted in various countries, the percentages of proven viral infections in cases of ALRI varied greatly, i.e., 83.2% in China [17], 75.2% in Spain [18], 50.3% in Brazil [19], and 36.7% in Turkey [20], due to differences in climatic and geographic conditions, the study population, and the range of respiratory agents investigated. In our population, the proportion of viral infections in the cases of bronchiolitis

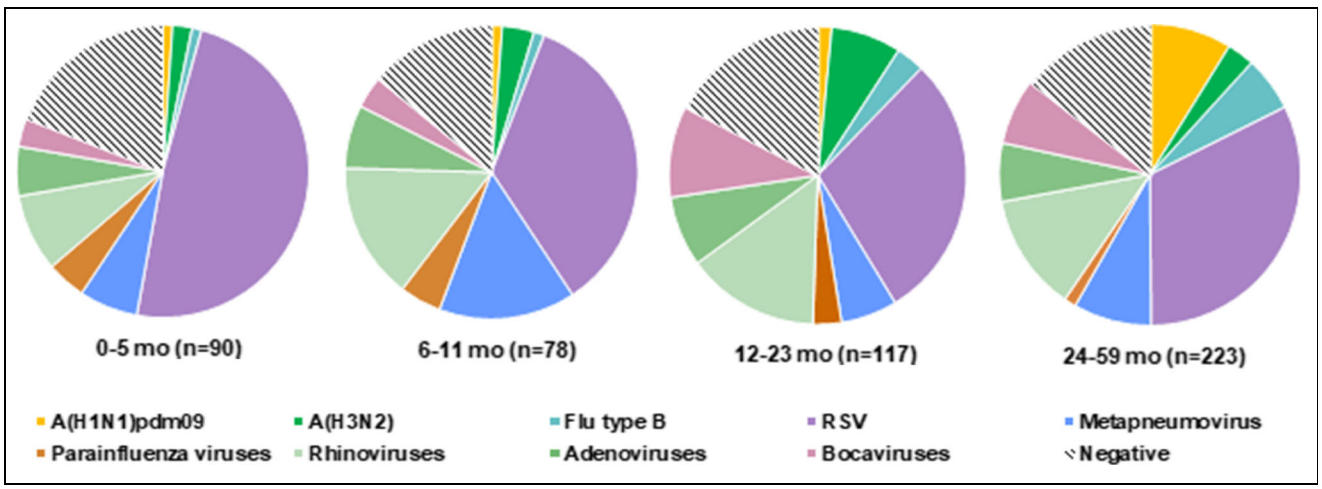

Fig. 3 Age distribution of children with detected respiratory viruses. Positive cases represent the sum of single infections and co-infections for each virus

Table 2 Prevalence of viral infections in children with acute lower respiratory tract diseases

	Bronchiolitis				Pneumonia			
	Total ($n = 328$)	Single (n, %)	Co-detection (n, %)	p	Total ($n = 187$)	Single (n, %)	Co-detection (n, %)	p
Demographic characteristics								
Median age in months	20				20			
Gender, male/female ratio	199/129			0.7732	107/80			
Hospitalizations, n (%)	323 (98.5)				186 (99.5)			
Hospitalization in ICU, n (%)	23 (7.0)				3 (1.6)			
Seasons ($n = 226$ in 2017)	151 (66.8)				75 (33.2)			
Winter	71 (47)	46 (64.8)	17 (23.9)		39 (52)	25 (64.1)	9 (23.1)	
Spring	29 (19.2)	21 (72.4)	2 (6.9)		19 (25.3)	8 (42.1)	4 (21.1)	
Summer	13 (8.6)	10 (76.9)	1 (7.7)		0			
Fall	38 (25.2)	25 (65.8)	6 (15.8)		17 (22.7)	6 (35.3)	1 (5.9)	
Viral etiology, n (%)	271 (82.6)	226 (68.9)	45 (13.7)		131 (70.1)	112 (59.9)	19 (10.2)	
Influenza A(H1N1)pdm09	11 (3.4)	10 (3.0)	1 (0.3)	0.6976	14 (7.5)	14 (7.5)	0	0.22
Influenza A(H3N2)	6 (1.8)	3 (0.9)	3 (0.9)	0.0596	16 (8.6)	10 (5.3)	6 (3.2)	*0.0134*
Influenza type B	11 (3.4)	9 (2.7)	2 (0.6)	1.000	10 (5.3)	8 (4.3)	2 (1.1)	0.6380
Respiratory syncytial virus	146 (44.5)	115 (35.1)	31 (9.5)	*0.0330*	47 (25.1)	35 (18.7)	12 (6.4)	*0.01*
Metapneumovirus	30 (9.1)	25 (7.6)	5 (1.5)	1.000	17 (9.1)	13 (7.0)	4 (2.1)	0.2716
Parainfluenza virus	13 (4)	9 (2.7)	4 (1.2)	0.2405	2 (1.1)	1 (0.5)	1 (0.5)	0.27
Rhinovirus	54 (16.5)	37 (11.3)	17 (5.2)	*0.0020*	17 (9.1)	14 (7.5)	3 (1.6)	0.7133
Adenovirus	15 (4.6)	5 (1.5)	10 (3.0)	*0.0001*	21 (11.2)	12 (6.4)	9 (4.8)	*0.0004*
Bocavirus	29 (8.8)	15 (4.6)	14 (4.3)	*0.0001*	7 (3.7)	5 (2.7)	2 (1.1)	0.2681

P values < 0.05 are indicated in italic

and pneumonia was 82.6% and 70.1%, respectively. These results are in agreement with previous reports in which viral pathogens were proven in over 90% of bronchiolitis cases [21–23] and in 43–81% of pneumonia cases [24–28]. Not surprisingly, RSV was the most frequently detected etiologic agent of ALRI, identified in 37.5% of the cases (44.5% in patients with bronchiolitis and 25.1% in patients with pneumonia), with a predominance of RSV subgroup B during the three seasons. RSV is known as a major pathogen in pediatric ALRIs, causing 50–90% of hospitalizations for bronchiolitis, 5–40% of those for pneumonia, and 10–30% of those for tracheobronchitis [23, 25, 29]. Consistent with other studies, the incidence of RSV infections was highest in the youngest age groups (< 6 months), which indicates higher susceptibility of this age group to RSV infection, and decreased with increased age due to the development of immunity after repeated infections [30]. The likelihood of occurrence of RSV infection immediately after birth points to the need for the administration of a RSV vaccine during the last months of pregnancy [31]. In the past decade, RSV treatments in development have included 10 vaccines and 11 therapeutic agents in active clinical trials [32].

Children aged < 5 years have high rates of influenza morbidity, and influenza-related complications and hospitalizations [33, 34]. In this study, influenza viruses were frequently detected, accounting for 13.2% of the cases. Their incidence rate was higher in the cases of pneumonia (21.4%) than in the cases of bronchiolitis (8.5%) ($p < 0.05$). With respect to the studied respiratory agents, specific prevention measures, such as vaccination and antiviral treatment, are only available against influenza viruses. Influenza vaccination would reduce greatly the burden of ALRI and prevent the severe forms of illness and deaths, but vaccine coverage in Bulgaria is extremely low (2–3% of the total population).

Human RVs are thought to cause a mild common cold, but they have been increasingly recognized as a significant pathogen of ALRIs in young children [10, 27, 35]. In a multicenter study involving 2207 children with severe bronchiolitis, RVs were reported as the second most common cause of bronchiolitis after RSV, accounting for 25.6% of the cases [21]. In another US study, among the 2358 children with pneumonia, RVs were detected in 27% of children [27]. In our data, RVs were also the second most common viruses in patients with bronchiolitis, detected in 16.5% of the cases (11.3% as a single pathogen).

In the literature, hMPV is a common pathogen in pediatric ALRIs and is second only to RSV as a causative agent of bronchiolitis in early childhood [36, 37]. HMPV incidence

has also been variable in different countries, as it may vary from year to year in the same region. In this study, hMPV was the third most frequent agent of bronchiolitis, identified as a single pathogen in 7.6% of the cases. The total hMPV detection rate of 9.1% was greater than that reported in studies in Spain (5.5%) [18] and China (6.5%) [38], and less than the 20% reported in a USA study [39]. According to other studies, children with single hMPV infections were older compared to those with single RSV infections (21 mo vs 19 mo) [7].

Studies of childhood ALRIs have reported various detection rates for AdV, i.e., 17% in Spain [40], 15.8% in Brazil [19], 11% in the USA [27], and 5.8% in China [41]. The proportion of AdV infections found among Bulgarian ALRI children was 7%. AdVs were associated with pneumonia and bronchiolitis as a sole pathogen in 6.4% and 1.5% of the cases, respectively. The high frequency (52.8%) of co-infections involving AdV can be explained by their long shedding period.

Human PIV types 1, 2, 3, and 4 have different clinical and epidemiological characteristics, and the most common cause of ALRI is PIV-3 [42]. In this study, PIVs occupied a small share in the etiological structure of ALRI—they were identified in 2.9% of the studied children, with a predominance of PIV-3 (2%). Only one child (0.2%) was PIV-2 positive. This PIV positive rate was lower than that reported in a study in Mexico (5.5%) of 1404 children under 5 years of age with pneumonia [26] and in a study in China (19.58%) of 771 children with ALRI [43].

Although BoVs have been identified in a significant percentage of children with ALRI in a number of countries [24, 44], their etiological role has not been elucidated yet. The high incidence (up to 75%) of co-infections involving BoV, as well the high frequency of BoV detection in asymptomatic children, casts doubts on their clinical significance and role as true pathogens [12]. In this first Bulgarian study of BoV infections, the incidence of BoVs among children with ALRI was 7%. They were detected as a single pathogen in 4.6% and 2.7% of the children with bronchiolitis and pneumonia, respectively. BoVs were also associated with a high rate (44.4%) of co-infections.

In a systematic review, Goka et al. reported that the incidence of mixed viral infections ranged from 5 to 62%, with a mean of 23% [45]. In this study, the proportion of cases with co-detections was 15.9%. The most common participants in mixed infections were AdV, BoV, A(H3N2), PIV, and RV in which the proportions of co-infections were 52.8%, 44.4%, 40.9%, 33.3%, and 28.2%, respectively. The simultaneous presence of more than one virus in the same sample is difficult to interpret. Highly sensitive PCR assays make it possible to identify very small amounts of viral nucleic acids, which are present during the incubation period or the convalescence phase. The identification of more than one virus can be explained by the prolonged shedding of virus that caused a previous infection, coincidental upper airway infection, or asymptomatic circulation of some viruses. Prolonged BoV shedding has been reported for up to 4.5 months in hospitalized children [46]. Due to the frequent detection of BoV, RV, AdV, coronaviruses, and enteroviruses in asymptomatic healthy children, their PCR results should be interpreted with caution, especially in the cases of co-detection with other potential pathogens [12]. In contrast, RSV, influenza viruses, hMPV, and PIV are substantially more frequent in patients with pneumonia than in controls, and their PCR detection is likely to be causative of disease [47–49]. The use of quantitative PCR with assessment of viral load could be helpful for the interpretation of multiple positive PCR results. The clinical significance of mixed infections is still unclear. There are controversial views on the relationship between multiple pathogen infections and disease severity. A few studies have reported that there is no relationship between mixed viral infections and illness severity [50], while others suggest that co-infections could be associated with a more severe disease course compared to single infections [51].

In our study, there were some variations in the timing of circulation of the individual viruses. Information about the seasonal distribution of respiratory viruses is important for enacting prophylactic measures among high-risk children and for strengthening control measures to prevent nosocomial infections.

The limitation of this study is that it did not involve testing for human coronaviruses, which can also cause ALRI [24, 27].

Conclusions

This study provides important information concerning the participation of the most common respiratory viruses in the development of ALRI in early childhood. It demonstrates the leading role of RSV as a causative agent of bronchiolitis and pneumonia in infants and young children. Our results highlight the importance of enhanced surveillance of respiratory viruses and indicate the urgent need for developing effective prevention strategies and new drug treatments.

Authors' contributions NK conceptualized and designed the study; ITz, SM, SV, and II were responsible for patient enrollment, assessment, and selection; NK, SA, ITr, IG, and SV performed the experiments; NK, ITr, ITz, SM, and II analyzed the work results; NK wrote the manuscript; TT and PP reviewed the final manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

This study was carried out in accordance with *The Code of Ethics of the World Medical Association* (Declaration of Helsinki) for experiments involving humans http://www.healthscience.net/resources/declaration-of-helsinki.

Conflict of interest The authors declare that they have no conflicts of interest relevant.

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References

1. Nair H, Simões EA, Rudan I et al (2013) Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis. Lancet 381(9875):1380–1390
2. Wishaupt JO, Versteegh FG, Hartwig NG (2015) PCR testing for paediatric acute respiratory tract infections. Paediatr Respir Rev 16(1):43–48
3. Pavia AT (2011) Viral infections of the lower respiratory tract: old viruses, new viruses, and the role of diagnosis. Clin Infect Dis 52(Suppl 4):S284–S289
4. WHO (2012) Vaccines against influenza WHO position paper – November 2012. Wkly Epidemiol Rec 47(87):461–476
5. Nair H, Brooks WA, Katz M et al (2011) Global burden of respiratory infections due to seasonal influenza in young children: a systematic review and meta-analysis. Lancet 378(9807):1917–1930
6. Shi T, McAllister DA, O'Brien KL et al (2017) Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: a systematic review and modelling study. Lancet 390(10098): 946–958
7. Moe N, Krokstad S, Stenseng IH et al (2017) Comparing human Metapneumovirus and respiratory syncytial virus: viral co-detections, genotypes and risk factors for severe disease. PLoS One 12(1):e0170200
8. Papenburg J, Hamelin ME, Ouhoummane N et al (2012) Comparison of risk factors for human metapneumovirus and respiratory syncytial virus disease severity in young children. J Infect Dis 206:178–189
9. Thomazelli LM, Oliveira DBL et al (2017) Human parainfluenza virus surveillance in pediatric patients with lower respiratory tract infections: a special view of parainfluenza type 4. J Pediatr (Rio J)
10. Jacobs SE, Lamson DM, St George K, Walsh TJ (2013) Human rhinoviruses. Clin Microbiol Rev 26(1):135–162
11. Echevarría M (2008) Adenoviruses in immunocompromised hosts. Clin Microbiol Rev 21:704–715
12. Longtin J, Bastien M, Gilca R et al (2008) Human bocavirus infections in hospitalized children and adults. Emerg Infect Dis 14(2): 217–221
13. CDC protocol of real-time RTPCR for influenza A(H1N1). Geneva: World Health Organization. 2009. http://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf?ua=1
14. Kodani M, Yang G, Conklin LM et al (2011) Application of TaqMan low-density arrays for simultaneous detection of multiple respiratory pathogens. J Clin Microbiol 49(6):2175–2182
15. Lu X, Chittaganpitch M, Olsen SJ et al (2006) Real-time PCR assays for detection of bocavirus in human specimens. J Clin Microbiol 44:3231–3235
16. Zlateva KT, Vijgen L, Dekeersmaeker N et al (2007) Subgroup prevalence and genotype circulation patterns of human respiratory syncytial virus in Belgium during ten successive epidemic seasons. J Clin Microbiol 45(9):3022–3030
17. Zhou JY, Peng Y, Peng XY et al (2018 Mar) Human bocavirus and human metapneumovirus in hospitalized children with lower respiratorytract illness in Changsha, China. Influenza Other Respir Viruses 12(2):279–286
18. García-García ML, Calvo C, Rey C et al (2017) Human metapnuemovirus infections in hospitalized children and comparison with other respiratory viruses. 2005–2014 prospective study. PLoS One 12(3):e0173504
19. Ferone EA, Berezin EN, Durigon GS et al (2014) Clinical and epidemiological aspects related to the detection of adenovirus or respiratory syncytial virus in infants hospitalized for acute lower respiratory tract infection. J Pediatr (Rio J) 90:42–49
20. Hatipoğlu N, Somer A, Badur S et al (2011) Viral etiology in hospitalized children with acute lower respiratory tract infection. Turk J Pediatr 53(5):508–516
21. Mansbach JM, Piedra PA, Teach SJ et al (2012) Prospective multicenter study of viral etiology and hospital length of stay in children with severe bronchiolitis. Arch Pediatr Adolesc Med 166(8):700–706
22. Janahi I, Abdulkayoum A, Almeshwesh F et al (2017) Viral aetiology of bronchiolitis in hospitalised children in Qatar. BMC Infect Dis 17(1):139
23. Calvo C, Pozo F, García-García ML et al (2010) Detection of new respiratory viruses in hospitalized infants with bronchiolitis: a three-year prospective study. Acta Paediatr 99(6):883–887
24. García-García ML, Calvo C, Pozo F et al (2012) Spectrum of respiratory viruses in children with community-acquired pneumonia. Pediatr Infect Dis J 31(8):808–813
25. Esposito S, Daleno C, Prunotto G et al (2013) Impact of viral infections in children with community-acquired pneumonia: results of a study of 17 respiratory viruses. Influenza Other Respir Viruses 7(1):18–26
26. Wong-Chew RM, García-León ML, Noyola DE et al (2017) Respiratory viruses detected in Mexican children younger than 5 years old with community-acquired pneumonia: a national multicenter study. Int J Infect Dis 62:32–38
27. Jain S, Williams DJ, Arnold SR et al (2015) Community acquired pneumonia requiring hospitalization among U.S. children. N Engl J Med 372(9):835–845
28. Jiang W, Wu M, Zhou J et al (2017) Etiologic spectrum and occurrence of coinfections in children hospitalized with community-acquired pneumonia. BMC Infect Dis 17(1):787
29. Hall CB (2001) Respiratory syncytial virus and parainfluenza virus. N Engl J Med 344(25):1917–1928
30. Feng L, Li Z, Zhao S et al (2014) Viral etiologies of hospitalized acute lower respiratory infection patients in China, 2009-2013. PLoS One 9(6):e99419
31. Saso A, Kampmann B (2016) Vaccination against respiratory syncytial virus in pregnancy: a suitable tool to combat global infant morbidity and mortality? Lancet Infect Dis 16(8):e153–e163
32. Mazur NI, Martinón-Torres F, Baraldi E et al (2015) Lower respiratory tract infection caused by respiratory syncytial virus: current management and new therapeutics. Lancet Respir Med 3(11):888–900
33. Huai Y, Guan X, Liu S et al (2017) Clinical characteristics and factors associated with severe acute respiratory infection and influenza among children in Jingzhou, China. Influenza Other Respir Viruses 11(2):148–156
34. Lafond KE, Nair H, Rasooly MH et al (2016) Global role and burden of influenza in pediatric respiratory hospitalizations, 1982-2012: a systematic analysis. PLoS Med 13(3):e1001977

35. Zeng SZ, Xiao NG, Xie ZP et al (2014) Prevalence of human rhinovirus in children admitted to hospital with acute lower respiratory tract infections in Changsha, China. J Med Virol 86(11): 1983–1989
36. García-García ML, Calvo C, Rey C et al (2017) Human metapneumovirus infections in hospitalized children and comparison with other respiratory viruses. 2005–2014 prospective study. PLoS One 12(3):e0173504
37. Edwards KM, Zhu Y, Griffin MR et al (2013) Burden of human metapneumovirus infection in young children. N Engl J Med 368(7):633–643
38. Xiao NG, Zhang B, Xie ZP et al (2013) Prevalence of human metapneumovirus in children with acute lower respiratory infection in Changsha, China. J Med Virol 85(3):546–553
39. Williams JV, Harris PA, Tollefson SJ et al (2004) Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N Engl J Med 350(5):443–450
40. Calvo C, García-García ML, Sanchez-Dehesa R et al (2015) Eight year prospective study of adenoviruses infections in hospitalized children. comparison with other respiratory viruses. PLoS One 10(7):e0132162
41. Liu C, Xiao Y, Zhang J et al (2015) Adenovirus infection in children with acute lower respiratory tract infections in Beijing, China, 2007 to 2012. BMC Infect Dis 15:408
42. Liu WK, Liu Q, Chen DH et al (2013) Epidemiology and clinical presentation of the four human parainfluenza virus types. BMC Infect Dis 13:28
43. Xiao NG, Duan ZJ, Xie ZP et al (2016) Human parainfluenza virus types 1-4 in hospitalized children with acute lower respiratory infections in China. J Med Virol 88(12):2085–2091
44. Ghietto LM, Cámara A, Zhou Y et al (2012) High prevalence of human bocavirus 1 in infants with lower acute respiratory tract disease in Argentina, 2007–2009. Braz J Infect Dis 16(1):38–44
45. Goka EA, Vallely PJ, Mutton KJ, Klapper PE (2014) Single and multiple respiratory virus infections and severity of respiratory disease: a systematic review. Paediatr Respir Rev 15(4):363–370
46. Blessing K, Neske F, Herre U et al (2009) Prolonged detection of human bocavirus DNA in nasopharyngeal aspirates of children with respiratory tract disease. Pediatr Infect Dis J 28(11):1018–1019
47. Self WH, Williams DJ, Zhu Y et al (2016) Respiratory viral detection in children and adults: comparing asymptomatic controls and patients with community-acquired pneumonia. J Infect Dis 213(4): 584–591
48. Shi T, McLean K, Campbell H, Nair H (2015) Aetiological role of common respiratory viruses in acute lower respiratory infections in children under five years: a systematic review and meta-analysis. J Glob Health 5(1):010408
49. Rhedin S, Lindstrand A, Rotzén-Östlund M et al (2014) Clinical utility of PCR for common viruses in acute respiratory illness. Pediatrics 133(3):e538–e545
50. Asner SA, Rose W, Petrich A et al (2015) Is virus coinfection a predictor of severity in children with viral respiratory infections? Clin Microbiol Infect 21:264.e1–264.e6
51. Harada Y, Kinoshita F, Yoshida LM et al (2013) Does respiratory virus coinfection increases the clinical severity of acute respiratory infection among children infected with respiratory syncytial virus? Pediatr Infect Dis J 32(5):441–445