



INFECTIONS WITH INFLUENZA VIRUSES, RESPIRATORY-SYNCYTIAL VIRUS AND HUMAN METAPNEUMOVIRUS AMONG HOSPITALIZED CHILDREN AGED ≤ 3 YEARS IN BULGARIA

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ABSTRACT

PURPOSE: Influenza viruses, respiratory-syncytial virus (RSV) and human metapneumovirus (HMPV) are among the most common causes of upper and lower respiratory tract infections in infants and young children worldwide. The aim of this study was to determine the contribution of these respiratory pathogens in cases of severe acute respiratory tract infections among hospitalized children aged ≤ 3 years during the 2013/2014 season in Bulgaria.

METHODS: Nasopharyngeal swabs from 168 children hospitalized for acute respiratory tract infections in different regions of country were tested for influenza A/B viruses with Real Time RT-PCR. Influenza virus negative samples were examined for RSV and HMPV.

RESULTS: A total of 63 (38%) samples were influenza virus positive: 46 (73%) for pandemic virus A(H1N1)pdm09; 13 (21%) for A(H3N2). All 105 negative samples were analyzed for RSV and HMPV with Real Time RT-PCR. RSV was detected in 16 (15%) while HMPV- in 13 (12%) specimens. Co-infection RSV+HMPV was found in one patient. The contribution of influenza viruses, RSV and HMPV in cases of bronchiolitis, pneumonia and CNS infections was determined.

CONCLUSIONS: Influenza viruses were the most frequent viral pathogens causing severe respiratory tract infections among hospitalized children ≤ 3 years of age during the 2013/ 2014 season in Bulgaria.

Key words: A(H1N1)pdm09, A(H3N2), bronchiolitis, pneumonia

INTRODUCTION

Acute respiratory infections occupy the largest share in the structure of infectious morbidity and mortality among children < 5 years worldwide (1, 2). They are associated with an enormous number of outpatient visits, hospitalizations and represent a substantial health care burden. Among the many viruses (above 200) that cause diseases of the respiratory tract, the members of

the family Orthomyxoviridae (influenza viruses) and the family Paramyxoviridae (RSV, HMPV, parainfluenza viruses) are the most common causes (3). Children under 3 years are particularly vulnerable to these viral pathogens as a result they suffer more often, the disease occurs more severe, with frequent involvement of the lower respiratory tract (4, 5, 6). Patients infected by diverse respiratory viruses exhibit overlapping symptoms that make clinical diagnosis unreliable. The development and widespread use of molecular techniques in recent years allow more accurate and complete study the role of these and other pathogens (viral, bacterial, parasitic) in the etiology of acute respiratory infections. Compared with

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conventional methods for the diagnosis of respiratory infections (viral cultures, antigen detection), molecular methods are characterized by the highest sensitivity and specificity (7). They offer fast and accurate diagnosis, due to which it is possible to undertake adequate treatment, including avoiding unnecessary treatment with antibiotics.

Three types (A, B and C) of *influenza viruses* circulate in the human population, types A and B cause clinically important respiratory illness. Influenza viruses infect all age groups but infants and young children have higher risk of severe disease and /or serious complications.

RSV is a leading cause of pediatric viral respiratory tract infections. Children have a 50% chance of being infected with RSV during the first year of their life. By 2-3 years of age, virtually all children have been infected at least once. Clinical manifestations of RSV infection are various and include fever, cough, rhinorrhea, croup, wheezing, asthma, dyspnea and hypoxia (8, 9). The most common complications are tracheobronchitis, bronchiolitis and pneumonia. Among children, RSV infection is the cause of 50 to 90% of hospitalizations for bronchiolitis, 5 to 40% of those for pneumonia, and 10 to 30% of those for tracheobronchitis (10).

The signs and symptoms of *HMPV* infection are similar to those for RSV and include fever, rhinorrhea, cough, otitis media, wheezing, dyspnea and asthma exacerbation. *HMPV* is second only to RSV as cause of bronchiolitis in early childhood (8, 11).

The aim of this study was to determine the contribution of influenza viruses, RSV and *HMPV* in cases of severe acute respiratory tract infections among hospitalized children aged ≤ 3 years during the 2013/2014 winter season in Bulgaria.

MATERIALS AND METHODS

From October 2013 until April 2014 a total of 168 nasopharyngeal swabs from children aged ≤ 3 years hospitalized for influenza like illness (ILI) or acute respiratory disease (ARD) in different regions of country were tested for

influenza A/B viruses. Viral nucleic acids extracted from specimens using a iPrep Pure Link Virus Kit (Invitrogen) according to the manufacturer's instructions. Detection and typing/ subtyping of influenza viruses were carried out with Real Time RT-PCR kit - SuperScript III Platinum [®] One-Step Quantitative RT-PCR System (Invitrogen) using primers and probes donated by CDC Atlanta. Influenza virus negative samples were examined by individual singleplex Real Time RT-PCR using specific primers/probes for RSV and *HMPV*.

RESULTS

From 168 patients tested 90 (54%) were boys and 78 (46%) – girls. The age of studied patients was in range of 1 to 47 months with mean age of $23,74 \pm 12,69$. 58% (97/168) of children had diagnosis ILI or ARD. We analyzed some common complications of the lower respiratory tract (laryngotracheitis, bronchiolitis, pneumonia) and central nervous system (CNS) (febrile seizures, oedema cerebri, meningitis, encephalopathy, meningoencephalitis). The distribution of patients examined by age and clinical diagnosis is shown in **Table 1**.

Viral infections were laboratory confirmed in 92 (55%) samples. The remaining 76 (45%) specimens were negative for all respiratory viruses tested. Among the 92 positive samples, there were 2 (2%) samples with detected co-infections. Out of 168 samples tested, 63 (38%) were influenza virus positive. Influenza A(H1N1)pdm09 and A(H3N2) viruses were detected in 46 (73%) and 13 (21%) of samples, respectively; a mixture of both viruses was found in one sample. Three influenza type A viruses were unsubtypeable. Influenza type B viruses were not detected in examined children. Total of 105 influenza virus negative samples were tested for RSV and *HMPV*, that were detected in 16 (15%) and 12 (11%) samples, respectively. Dual infection RSV+*HMPV* was found in one sample.

In **Table 2** the distribution of detected respiratory viruses according to patient age is shown.

Table 1. Epidemiologic and clinical characteristic of patients examined / infected

	Total tested (n=168)	Infected patients (%)		
		Total infections (n=92)	Single infections (n=90)	Co-infections (n=2)
<i>Gender</i>				
Male	90	49	49	49
Female	78	43	41	2
<i>Age groups (months)</i>				
≤1	1	1	1	
2-3	6	4	4	
4-6	9	3	3	
7-12	26	12	12	
13-24	66	31	30	1
25-36	41	25	25	
37-47	19	16	15	1
<i>Clinical diagnosis</i>				
ILI/ARD	97	46	45	1
Laryngotracheitis	6	6	6	
Bronchiolitis	9	7	7	
Pneumonia	28	20	19	1
Neuroinfection	28	13	13	

Table 2. Distribution of detected respiratory viruses according to patient age

Viral etiology	Total positive	Age groups (months)						
		≤1	2-3	4-6	7-12	13-24	25-36	37-47
Influenza A(H1N1)pdm09	46	0	3	2	5	16	13	7
Influenza A(H3N2)	13	0	0	0	2	3	6	2
A(H1N1)pdm09+ A(H3N2)	1	0	0	0	0	0	0	1
Influenza type A	3	0	0	1	0	1	1	0
RSV	16	1	0	0	2	5	4	4
HMPV	12	0	1	1	2	6	2	0
RSV + HMPV	1	0	0	0	0	0	0	1
Total	92	1	4	4	11	31	26	15

Respiratory viruses occurred in all age groups. Incidence of influenza A(H1N1)pdm09 and A(H3N2) infections was larger in age groups 13-24 and 25-36 months, respectively. RSV and HMPV were the most frequently detected in age groups 13-24 months. Co-infections were found

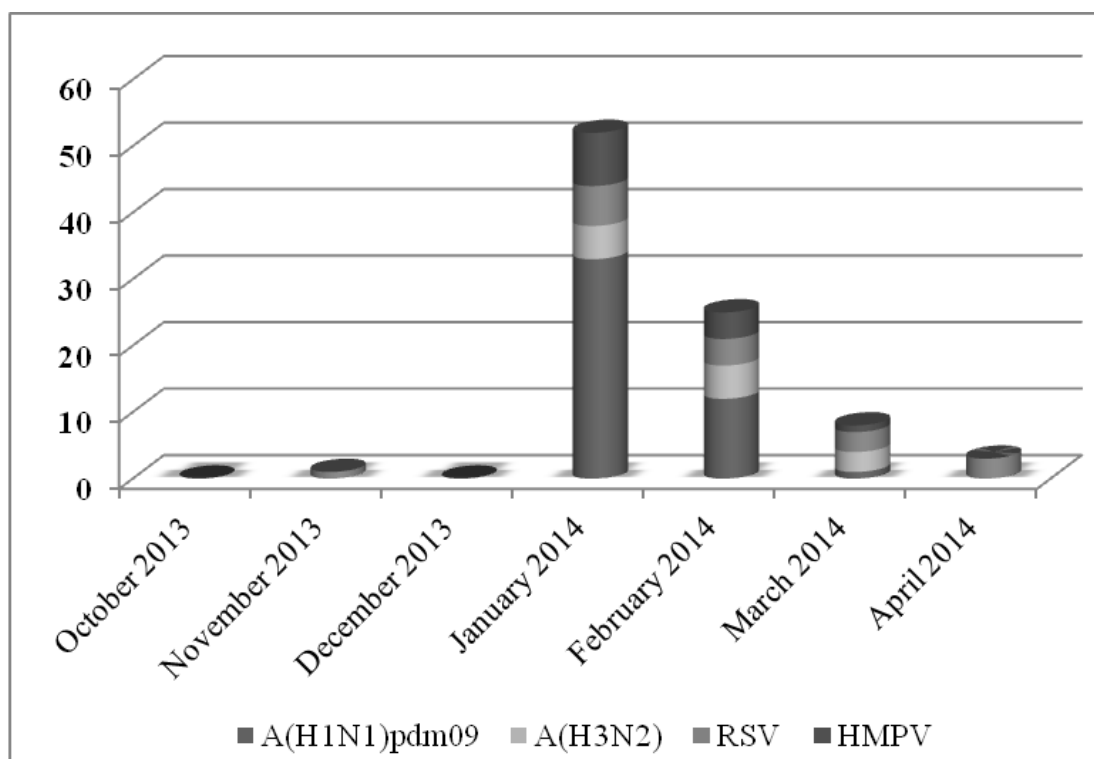
in children aged 37-47 months. The largest number of respiratory viruses was identified in age groups 13-24 and 25-36 months. Median age of children infected by different respiratory viruses and ratio male/female is shown in **Table 3**.

Table 3. Median age and gender distribution of children with detected respiratory viruses

	Detected respiratory viruses			
	A(H1N1)pdm09	A(H3N2)	RSV	HMPV
Median age (months)	25,52 ±13,01	29,23±11,31	27,76±14,48	19,69±11,67
Male / female	23 / 23	8 / 5	6 / 9	10 / 2

Children with HMPV infections had the least median age. Among positive patients 49 (53%) were male and 43 (47%) – female. Boys were more frequently infected by influenza A(H3N2) and HMPV in comparison with the girls. Ratio male: female was larger in patients with HMPV infection. RSV infections were more prevalent among girls.

Figure 1 illustrates the monthly distribution of the individual respiratory viruses investigated from October 2013 through April 2014. The largest number of respiratory viruses was identified in February and March. Influenza viruses and HMPV were detected from January to March, RSV from November to April.

**Figure 1.** Monthly distribution of the individual respiratory virus infections

Viral respiratory infections in early childhood sometimes occur with complications, the most common of which include involvement of the lower respiratory tract or CNS (3). We have analyzed the contribution of the influenza viruses, RSV and HMPV in development of the

afore mentioned complications. **Table 4** presents the clinical diagnoses of patients infected with different respiratory viruses. Median age of children and male/female ratio in groups with different clinical diagnosis are also shown.

Table 4. Distribution of clinical diagnoses

	Clinical diagnoses				
	Laryngo- tracheitis (n=6)	Bronchioliti s (n=9)	Pneumonia (n=28)	Neuro- infections (n=28)	ILI/ARD (n=97)
Age (months)	24,83±18,48	17,44±14,55	26,75±12,12	19,25±14,18	24,73±11,49
Male / female gender	2 / 4	4 / 5	14 / 14	13 / 15	54 / 43
Influenza A(H1N1)pdm09	5	1	11	8	21
Influenza A(H3N2)	0	0	2	2	9
A(H1N1)pdm09 + A(H3N2)	0	0	1	0	0
Influenza type A	0	0	1	0	2
RSV	0	4	1	1	10
HMPV	1	2	4	2	3
RSV + HMPV	0	0	0	0	1
Total	6	7	20	13	46

Median age of patients with bronchiolitis and CNS infections was lower in comparison with the median age in other groups ($P < 0,005$). Girls suffered more frequently by laryngotracheitis, bronchiolitis and CNS infections. ILI/ARD were more prevalent among boys. The proportion of positive samples among patients with laryngotracheitis was 6/6 (100%); bronchiolitis - 7/9 (78%); pneumonia - 20/28 (71%), neuroinfections - 13/28 (46%), ILI/ARD – 46/97 (47%). Among children with bronchiolitis RSV and HMPV were more prevalent. The most frequently cause of pneumonia and neuroinfections was influenza A(H1N1)pdm09 virus. This virus was detected in two children with meningitis and one child with meningoencephalitis. Influenza A virus was identified in one fatal case (child 6 months of age with pneumonia). Co-infections were proved in one case of pneumonia and one case of ILI.

DISCUSSION

In this paper we present the results of study of influenza virus, RSV and HMPV infections among hospitalized children ≤ 3 years during the 2013/2014 winter season in Bulgaria. Because of the important clinical and epidemiological importance of the indicated viral infections in many countries around the world are conducting

studies of their distribution and participation in the etiology of respiratory diseases. The widespread use of molecular techniques in recent years helped to clarify more precisely and completely the role of respiratory viruses in infectious pathology in childhood. In our study, carried out by singleplex Real-Time RT-PCR, a viral etiology was demonstrated in 55% of cases. The remaining 45% of the cases may have been caused by other viruses (adenovirus, coronavirus, rinovirus bocavirus, etc.) or bacteria that should be further investigated. Detection rate of mixed infections was very low (2%). Several researchers have reported co-infection rates of approximately 10-30% (12). Some studies have suggested that co-infections are associated with more severe symptoms than single infections. The clinical significance of mixed infections with respect to causing more severe illness in our study is not clear. Dual infections were demonstrated only in one cases of pneumonia and in one case of ILI.

Detection rate of individual respiratory viral pathogens in different studies varies significantly, which can be explained by variability of climatic conditions in different countries, age groups covered in the analyses, detection methods etc. (12, 13, 6, 5, 14). In our

study distribution of viruses detected was 27%, 8%, 15% and 12% for A(H1N1)pdm09, A(H3N2), RSV and HMPV, respectively. Influenza A(H1N1)pdm09 virus had the most significant contribution.

Influenza epidemics are characterized by temporal and geographical variations in the distribution of the different types / subtypes influenza viruses, by their virulence, clinical activity and the characteristics of the epidemic process. In Bulgaria influenza 2013/2014 season was characterized by a higher incidence rate compared to the previous two seasons, shorter duration and moderate intensity. This season co-circulated all seasonal influenza viruses as A(H1N1)pdm09 virus was predominant and accounted for 63% of all proven influenza viruses. Influenza type B viruses were very rarely detected. In present study involving children aged ≤ 3 years pandemic virus was the most frequently proved. Among the detected total 63 influenza viruses, A(H1N1)pdm09 were 46 (73%) and A(H3N2) – 13 (28%). A mixed infection A(H1N1)pdm09 + A(H3N2) was detected in a child with pneumonia. The average age of children with proven A (H1N1) pdm09 and A(H3N2) virus infection was $25,52 \pm 13,01$ and $29,23 \pm 11,31$ months respectively. Influenza A(H1N1)pdm09 were the most frequently detected virus among patients with pneumonia and neuroinfections.

The positive rate of RSV infection varies in different countries (9, 14, 15, 16). In our previous study involving children aged ≤ 1 year suffering from bronchiolitis or pneumonia in the 2010/2011 season, the incidence of RSV infection was 39,8% and 39,2%, respectively (17). In present study it was lower - 16%. A possible reason for this is that the scope of the first study was the most vulnerable child age and clinical syndromes bronchiolitis and pneumonia in which the RSV is the leading etiological agent. It is known that RSV causes severe infections of the lower respiratory tract most commonly in children younger than one year, especially those less than 6 months, and male patients appeared to have higher rates of infection than females. In our study the average age of children with proven RSV infection was $27,76 \pm 14,48$ months, indicating that RSV causes severe disease requiring hospital treatment also in the older children. Girls were more frequently

infected than boys. Among patients with bronchiolitis RSV was proven the most frequently. It was identified in 44% of the studied cases of bronchiolitis, 4% of the cases of pneumonia and 4% of cases of neuroinfections. RSV was detected in a child with pleuropneumonia treated in intensive care unit.

HMPV is closely related to RSV and has similar epidemiological and clinical characteristics (8, 11, 18). Detection rate of HMPV have been also variable in different countries (19). In our study it was detected in 13 children. HMPV was etiological agent of 22%, 14% and 14% of cases of bronchiolitis, pneumonia and CNS infections respectively.

Monthly distribution of proven respiratory viruses showed the highest detection rates in February and March that confirms winter seasonality of these infections (20). Due to the limited study period is not possible to draw conclusions about differences in the prevalence of various respiratory viruses.

This study is the first to Bulgaria, which analyzes simultaneously the participation of influenza viruses, RSV and HMPV in the etiology of severe respiratory disease among hospitalized children ≤ 3 years using molecular method Real Time RT-PCR. Limitation of this research is that it did not study other viral and bacterial pathogens and that it covered only one season. Nevertheless, this study has shown the leading role of influenza viruses and the significant contribution of RSV and HMPV in the development of serious respiratory disease in early childhood. It confirms the need for ongoing systematic investigation of viral respiratory pathogens with the extension of the research and the ultimate goal - to develop adequate strategies for therapeutic behavior and prevention.

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