VIRAL ETIOLOGY OF ACUTE RESPIRATORY INFECTIONS AMONG CHILDREN UNDER 5 YEARS OF AGE IN BULGARIA, DURING THE 2013/2014 AND 2014/2015 SEASONS

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ABSTRACT

PURPOSE: A wide range of different viruses cause respiratory tract diseases of varying severity. This study aims to determinate the contribution of nine different viral pathogens in cases of acute respiratory infections (ARI) in children aged less than 5 years, during the 2013/2014 and 2014/2015 winter seasons in Bulgaria.

METHODS: A total of **412** nasopharyngeal swabs of children under the age of 5 were tested. Children were either outpatient, or hospitalized for influenza-like illness. The clinical samples were tested for Orthomyxoviruses (influenza A/B), Paramyxoviruses (respiratory-syncytial virus – RSV; human metapneumovirus – HMPV; human parainfluenza viruses – HPIV, type 1/2/3) and human rhinoviruses (HRV) using Real Time RT-PCR reactions based on specific primers/probe.

RESULTS: Virus infections were confirmed in **258** (62.6%) samples. In 107 (26%) of samples influenza viruses were detected. All of the 306 influenza virus negative patients were analyzed for Paramyxo- and Rhinoviruses. Detection rate for RSV, HMPV, HPIV1/2/3 and HRV was 19%, 5.9%, 2%, 1.3%, 3.3% and 14.1%, respectively.

CONCLUSION: The present study is the first one in Bulgaria which analyzes the participation of nine different viral pathogens in the etiology of ARI among children below the age of 5 years. The study reveals the leading role of influenza viruses, RSV and rhinoviruses in the development of serious respiratory diseases in early childhood.

Key words: rhinovirus, acute respiratory infections, influenza virus, RSV, HMPV

INTRODUCTION

Respiratory viral infection is a major cause of morbidity and mortality among children. Infancy, in particular, is a time of increased disease susceptibility and severity. Early-life viral infection causes acute illness and can be associated with the development of wheezing and asthma in later life (1, 4). Acute respiratory infections are associated with an enormous number of outpatient visits and hospitalizations, and represent a substantial health care burden. A wide range of different viruses cause respiratory tract diseases of varying severity, as members of the family Orthomyxoviridae – influenza viruses, the family Paramyxoviridae - respiratory-syncytial

virus (RSV), human metapneumovirus (HMPV), human parainfluenza viruses (HPIV) and the Picornaviridae family - human rhinoviruses (HRV), are among the most common causative agents (2, 3).

Clinical manifestation in different viral infections is similar and nonspecific, and therefore etiological diagnosis based only on clinical parameters, is unreliable. The widespread use of molecular techniques in recent years allows full and accurate clarification of the role these and other pathogens (viruses, bacteria, and fungi) play in the etiology of acute respiratory diseases (5, 6). The aim of the present study is to determinate the contribution of nine different viral pathogens in cases of acute respiratory infections in children aged less than 5 years, during the 2013/2014 and 2014/2015 winter seasons in Bulgaria.

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Especially rhinovirus etiology in cases of influenza-like and ARI in children has not been studied yet in Bulgaria and there are no data on the circulation of these viruses and their role in the occurrence of ARI and their complications.

MATERIALS AND METHODS

A total of 412 children aged < 5 years, ambulatory treated or hospitalized for influenza like illness (ILI) or acute respiratory illness in different regions of the country were enrolled in the present study. Most of the patients (58%) presented without complications. The rest were complicated with bronchiolitis, pneumonia, febrile seizures, cerebral oedema, meningitis, encephalopathy, encephalitis, etc.

Extraction of nucleic acids and real time RT-PCR method

Viral nucleic acids were automatically extracted from respiratory specimens using a commercial ExiPrep Dx Viral DNA/RNA Kit (Bioneer) according to the manufacturer's instructions. Laboratory testing was conducted at the National Laboratory "Influenza and Acute Respiratory Diseases" recognized by WHO as a National Influenza Center. Detection and typing/subtyping of influenza viruses was carried out by RT-PCR method with the use of a kit - SuperScript III Platinum® One-Step Quantitative RT-PCR System (Invitrogen). All samples were initially tested for influenza A and B viruses using primers and probes donated by CDC Atlanta and those positive for influenza A were

subsequently tested for A(H1N1)pdm09 and A(H3N2). Influenza virus negative samples were examined by singleplex Real Time RT-PCRs using specific primers/probes for RSV, HMPV, HPIV 1/2/3 and HRV assays using the AgPath-ID One Step RT-PCR Kit (Applied Biosystems). Primers and probes used in the study were identical to those previously described (7).

RESULTS

The present study included two winter epidemic seasons in Bulgaria: from October 2013 to May 2014 and from October 2014 to May 2015. The study population consisted of 412 children aged < 5 years presenting with ILI or ARI. During the 2013/2014 season 203 children were tested, during the 2014/2015 season – 209; 139 (33.7%) among them were outpatient, and 273 (66.3%) – hospitalized. Age of the patients varied from 30 days to 60 months (average 21.53 ± 12.54 months). 229 (55%) were males, and 183 (45%) – females.

Virus infections were laboratory confirmed in 258 (62.6%) patients' samples. Monoinfections were identified in 245 (59.4%) patients. Thirteen (3.15%) patients were co-infected with two viruses. In 106 (26%) of patients' samples were detected influenza viruses (**Figure 1**). Influenza A(H1N1)pdm09 virus was confirmed in 54 (50.9%) of the samples, A(H3N2) type influenza virus was found in 41 (38.7%) of the samples, type B viruses were detected in 5 (4.7%) samples. Six influenza A viruses (5.7%) were unsubtypeable.

Figure 1. Confirmed Influenzavirus infections

All of the 306 influenza virus negative patients were tested for Paramyxo- and Rhinoviruses (**Figure 2**). Respiratory-syncytial virus was found in 58 (19.0%) of the samples. Human rhinovirus was the second most common detected virus in patients' samples. HRV was confirmed in 43 (14.05%) of the patients. In 18 (5.9%) samples human metapneumovirus was detected. Parainfluenza viruses type 1, 2 and 3 were identified in 6 (2%), 4 (1.3%) and 10 (3.3%) samples, respectively (**Table 1**).

Overall, the most frequently detected agent was influenza A(H1N1)pdm09, followed by RSV and HRV in single infections. In the 13 (3.15%) patients with co-infection the combination RSV + HMPV was found in 2 samples. Co-infections with RSV + HPIV1 and RSV+HPIV3 were identified in 2 and 2 samples respectively. One patient was infected with 2 types of human parainfluenza viruses (HPIV1+HPIV2) simultaneously and in 5 of the cases co-infection with RSV+HRV was detected.

Figure 2. Confirmed paramyxoviruses and rhinoviruses

Table 1. Distribution of detected respiratory viruses by seasons and in outpatients/inpatients.

	Number of detected respiratory viruses									
	Orthomyxoviruses				Paramyxoviruses					Rhino viruses
	A (H1N1) pdm	A (H3N2)	Type B	A	RSV	HMPV	PIV 1	PIV 2	PIV 3	RV
2013/14	44 (21,7%)	15 (7,4%)	0	6 (3%)	16 (11,6%)	17 (12,3%)	6 (4,3%)	2 (1,4%)	5 (3%)	13 (9,1%)
2014/15	10 (4,8%)	26 (12,4%)	5 (2,4%)	0	42 (25,1%)	1 (0,6%)	0	2 (1,2%)	5 (3%)	30 (18%)
Outpatient	12 (8,6%)	15 (10,8%)	2 (1,4%)	3 (2%)	12 (14,3%)	5 (6%)	1 (1,2%)	1 (1,2%)	3 (3,6%)	15 (17,8%)
Hospitalized	42 (15,5%)	26 (9,52%)	3 (1%)	3 (1%)	50 (22,5%)	13 (5,8%)	5 (2,3%)	3 (1,3%)	7 (3,2%)	28 (12,6%)

Clinical characteristics

In infants and young children respiratory viruses may cause complications to the lower respiratory tract or the CNS. The contribution of influenza viruses, RSV, HMPV, HPIV1/2/3 and rhinoviruses in the development of the aforementioned complications is analyzed. Tables 2 and 3 present the clinical diagnoses of patients infected with different viruses, and male/female ratio in the groups with different clinical diagnosis. The proportion of influenza

virus positive samples among patients with bronchiolitis was 10 (27.7%), pneumonia - 21(42.85%), neurological complications - 15 (25.9%) and ARI – 61 (22.67%) (**Table 2**) RSV was the most common virus identified in patients with bronchiolitis and pneumonia – 13 (48.1%) and 13 (37.1%), respectively. In patients, diagnosed with ARI the most frequently detected virus was HRV – 33 (16.75%) (**Table 3**).

Table 2. Distribution of detected influenza viruses according to clinical diagnosis of the patients.

	Clinical Diagnosis			
	Bronchiolitis (n=36)	Pneumonia (n=49)	CNS complications (n=58)	ARI (n=269)
♀ / ♂	18/18	25/24	31/27	155/114
A(H1N1)pdm09	9 (25%)	14 (28.6%)	6 (10.3%)	25 (9.3%)
A(H3N2)	-	5 (10.2%)	8 (13.8%)	28 (10.4%)
B	1 (3%)	1 (2%)	-	3 (1.1%)
A/unsubtypable	-	-	1 (1.7%)	5 (1.8%)
A(H1N1)pdm09+ A(H3N2)	-	1 (2%)	-	-
Total (n=106)	10 (27.7%)	21 (42.85%)	15 (25.9%)	61 (22.67%)

Table 3. Distribution of detected paramyxoviruses according to clinical diagnosis of the patients.

	Clinical diagnosis			
	Bronchiolitis (n=27)	Pneumonia (n=35)	CNS complications(n=46)	ARI (n=197)
♀ / ♂	12/15	15/20	20/26	96/101
RSV	13 (48,1%)	13 (37,1%)	5 (11%)	27 (13,7%)
HMPV	4 (14,8%)	3 (8,6%)	2 (2,1%)	9 (4,5%)
HPIV1	-	1 (2,85%)	1 (2,1%)	4 (2,03%)
HPIV2	1 (3,7%)	-	1 (2,1%)	2 (1%)
HPIV3	-	2 (5,7%)	1 (2,1%)	8 (4,6%)
HRV	2 (7,4%)	1 (2,85%)	6 (13%)	33 (16,75%)
RSV+HMPV	1 (3,7%)	-	-	1 (0,5%)
RSV+HPIV1	-	1 (2,85%)	-	1 (0,5%)
RSV+HPIV3	-	-	-	2 (1%)
HPIV1+HPIV2	-	-	-	1 (0,5%)
RSV+HRV	-	1 (2,85%)	1 (2,1%)	3 (1,5%)
Total (n=151)	21 (78%)	22 (62,9%)	17 (37%)	91 (46,19%)

DISCUSSION

Influenza viruses, RSV, HMPV, HPIV and rhinoviruses are important causative agents of ARI in infants and young children worldwide. In the present study, the virological and clinical features of medically attended ART in Bulgaria during two consecutive winter seasons are evaluated. The incidence of nine respiratory viral infections is determined and their participation in the development of lower respiratory tract diseases and CNS (Central Nervous System) complications is analyzed. Viral etiology is demonstrated in 63% of the cases which has been carried out using a singleplex RT-PCR. The remaining 37% of the cases may have been caused by other viruses (adenovirus, coronavirus, etc.) or bacteria and should be further investigated. This incidence rate is comparable to rates reported in other studies (36.6% - 60.6%) (8-10, 11-12). Co-infections in this study were identified in a relatively small percentage of cases (3.15%). According to various studies performed mainly using multiplex PCR, co-infections are common, occurring in 10 - 70% of ARI (13, 14). There is no consensus on the impact of co-infection on disease severity. Several author groups suggest that infections with two or more pathogens have been associated with incidence of more severe symptoms, particularly co-infections with RSV (1).

Influenza epidemics are characterized by temporal and geographical variations in the distribution of the different types/subtypes of influenza viruses, their virulence, clinical activity and the characteristics of the epidemic process (15). In Bulgaria, the 2013/2014 winter season was dominated by influenza

A(H1N1)pdm09 virus. In the next season A(H3N2) was the predominant circulating strain. Influenza viruses were the most common pathogens identified in this study, accounting for 41.1% of all viruses detected.

The incidence of RSV infection varies in different countries and years (16). In the present study, the total detection rate of RSV was 22.5% (58 patients) of all virus positive samples. It is known that RSV is the most important pathogen of the lower respiratory tract among infants and young children and is the major cause of bronchiolitis and pneumonia (17). In this study, 80.6% (50) of RSV detections were found in hospitalized children and the frequency of RSV infections in children with bronchiolitis and pneumonia was 48.1% and 37.1% respectively. In these clinical groups RSV was the most frequently proven causative agent.

The incidence of HMPV infections also varies in different countries. It may vary from year to year in the same region. In this study, HMPV virus was detected in 18 (5.9%) of children studied, which is consistent with the detection rate (4 - 16%) reported in other studies of similar design (18). The association of HMPV with acute bronchiolitis and pneumonia has been documented in many studies (19-21). We found that HMPV was an etiological agent in 14.8% of cases of bronchiolitis and 8.6% of pneumonia.

Human parainfluenza virus type 1, 2, 3, and 4 are distinguished by their clinical and epidemiological characteristics (22). In this report HPIV has been detected in 6.6% of the children examined. These findings are similar

to these in other studies (23). HPIV has low participation in cases with complications. They have been detected in a single case of bronchiolitis and in three pneumonia cases.

Human rhinoviruses (HRVs) — the most prevalent respiratory viruses — cause up to half of the common colds and impose a significant economic burden (24). HRVs have also been associated with bronchiolitis (25), pneumonia (26) and exacerbation of breathing difficulties in populations with underlying respiratory conditions, including asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD) (27-29). Prior to the era of molecular testing, rhinoviruses were considered to be a relatively mild pathogen of questionable importance. However, the promotion of fast and sensitive molecular techniques that readily identify HRV infection imposes the need for surveillance of the circulation of these viruses. In the present study HRV was detected in 33 (16.7%) of children, diagnosed with ARI. HRV was identified in 2 (7.4%) samples from children with bronchiolitis and in one case of pneumonia. It is important to mention that the combination between HRV and RSV was the most common one identified in the cases of co-infection.

The present study is the first one in Bulgaria which simultaneously analyzes the participation of influenza viruses, RSV, HMPV, HPIV type 1, 2 and 3 and rhinoviruses in the etiology of acute respiratory diseases among children under 5 years of age. This study provides important information concerning the major viral respiratory infections in Bulgaria. It reveals the leading role of influenza viruses, RSV and rhinoviruses in the development of serious respiratory diseases in early childhood. The results obtained in the study confirm the need of a systematic surveillance of viral respiratory infections, extending the research in order to develop effective strategies for control and prevention.

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