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PREVALENCE OF RESPIRATORY VIRUSES IN CASES OF PEDIATRIC ACUTE RESPIRATORY TRACT ILLNESSES IN BULGARIA

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Abstract: Acute respiratory illnesses (ARI) are the most prevalent infectious diseases in pediatrics. This study aimed to determinate the frequency and clinical significance of the most often respiratory viruses among children aged <5 years with ARI in Bulgaria during the 2016/17 winter season.

Nasopharyngeal specimens of 398 young children from different country regions were collected and tested using individual Real Time PCR assays for detection of influenza viruses A/B, respiratory-syncytial virus (RSV), metapneumovirus (MPV), parainfluenza viruses (PIV 1/2/3), rhinoviruses (RV), adenoviruses (AdV) and bocaviruses (BoV). Samples with negative results were tested for human coronaviruses, PIV4 and enteroviruses (EV) by Multiplex PCR method using a Seeplex RV15 ACE Detection kit.

A total of 365 respiratory viruses were identified using Real Time PCR and the numerical distribution of influenza A(H1N1)pdm09, A(H3N2) and B, AdV, RV, RSV, BoV, MPV, PIV 1, PIV 2, PIV 3 was obtained as: 4 (1,1%), 85 (23%), 4 (1,1%), 40 (11%), 34 (9%), 142 (39%), 12 (3,3%), 25 (7%), 0 (0%), 5 (1,4%), 15 (4,1%), respectively. Among the 91 samples tested by Multiplex PCR, coronaviruses NL63 and OC4, EV and PIV 4 were detected in 8%, 1% and 1% of specimens, respectively. Viral respiratory tract infections occurred mainly in December, January and February.

According to study results, RSV, influenza A(H3N2) and AdV were the most frequently detected viruses and affected at most 0-4 age group. The study highlights the necessity of routine surveillance of respiratory viruses in order to determine their spreading and participation in etiology of ARI

INTRODUCTION

Acute respiratory tract infections (ARTI) are associated with serious health and social consequences and represent the largest deal in the structure of infectious morbidity and mortality worldwide. Data for 2010 show 11.9 million cases of severe and 3 million cases of very severe lower respiratory tract illnesses requiring hospital treatment in children under the age of 5 years (Nair et al., 2010). It was found that about 80% of ARTI have a viral etiology (Mäkelä et al., 1998). Over 200 viruses cause respiratory tract diseases of varying severity: from mild respiratory symptoms to fatal pneumonia (Tregoning JS, et al., 2010 ; Raj VS et al., 2014; Pavia AT, et al., 2011). A large spectrum of viruses are associated with respiratory diseases and the major causative pathogens in this relation are: influenza viruses, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), human parainfluenza viruses (hPIV 1,2,3) human rhinoviruses (HRV), adenoviruses (AdV), bocaviruses (BoV). Young children and infants are more susceptible and sensitive to these pathogens. Infections during the early childhood occur in a more severe form and often result in complications with involvement of the lower respiratory tract and the nervous system requiring hospitalization. One of the common complications of viral disease and the leading infectious cause of mortality in young children is pneumonia (Walker et al., 2013) According to the WHO, in developed countries, one among 20 children under 5 years of age develops the disease every year (Rudan et al., 2004) Clinical manifestations in different viral infections are similar, nonspecific, and therefore the etiological diagnosis based only on clinical parameters is unreliable. Rapid and accurate diagnosis is important in terms of adequate therapeutic approach and timely preventive and anti-epidemic measures. The widespread use of molecular techniques in recent years allows full and accurate determination of the role of various respiratory pathogens in the etiology of acute respiratory disease (ARD) (Ali et al., 2013; Bicer et al., 2013; Buller, 2013).

In this regard, the present study aims to determine the engagement and clinical impact of influenza and other major respiratory viruses for the development of acute respiratory infections among children under 5 years of age during the winter season 2016/1017 in Bulgaria.

MATERIALS AND METHODS

A total of 398 children aged < 5 years, ambulatory treated or hospitalized for influenza like illness (ILI) or acute respiratory illness (ARI) in different regions of the country were examined. Most of the patients presented with respiratory virus were complicated with laryngotracheitis, bronchiolitis, pneumonia, febrile seizures, cerebral oedema, meningitis, encephalopathy, encephalitis, etc.

Extraction of nucleic acid, Real- time RT-PCR, Multiplex PCR methods

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 SuperScript III Platinum® One-Step Quantitative RT-PCR System kit (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 - PCR assays using specific primers/probes for RSV, HMPV, HPIV 1/2/3, HRV, AdV and BoV and the AgPath-ID One Step RT-PCR Kit (Applied Biosystems). Primers and probes used in the study were identical to those previously described (Kodani, M, et al., 2011).

Negative samples for the above-mentioned respiratory viruses were analyzed also for human coronaviruses (HCoV) (NL63 / OC43; 229E/HKU), enteroviruses (EV), parainfluenza virus (PIV4) performing the Multiplex PCR method by Seeplex RV15 ACE Detection Kit (fast-track diagnostic kit).

RESULTS AND DISCUSSION

The present study included 2016/2017 winter epidemic season in Bulgaria: from October 2016 to April 2017. The study population consisted of 398 children aged < 5 years presenting with ILI or ARI. Most of the patients 345 (86.7%) were hospitalized and 53 (13.3%) were outpatients. Age of children varied from 30 days to 60 months (average 21.53 ± 12.54 months). Males were 57%, and females 43%. Virus infections were laboratory confirmed in 327 (82%) patients' samples (Table 1). Monoinfections were identified in 320 (85.4%) patients. Fifty three (14%) patients were co-infected with two viruses and two with three viruses (RSV/ HRV/ A(H3N2); RSV/ AdV/ BoV). A total of 366 respiratory viruses were detected by Real Time PCR and the numerical distribution of A (H1N1)pdm09, A (H3N2), B, AdV, RV, RSV, HBoV, HMPV, PIV 1, PIV 2, PIV 3 was obtained as: 4 (1,1%), 85 (23%), 4 (1,1%), 40 (11%), 33 (9%), 142 (39%), 12 (3,3%), 25 (7%), 0 (0%), 5 (1,4%), 15 (4,1%), respectively (Table 1). Influenza A(H3N2) virus was predominant virus during the 2016/2017 winter season in Bulgaria and legally was detected in 91,4% of influenza positive samples. A(H1N1)pdm09 virus was confirmed in 4.3%, type B viruses - in 4.3%.

Table 1. Distribution of analysed respiratory viruses detected using Real- time RT-PCR / Multiplex PCR methods among hospitalized and ambulatory treated patients with ILI or ARI .

Number of detected respiratory viruses																
	Real time PCR method											Multiplex PCR method				Total number
	A(H1N1) pdm	A(H3N2)	B	RSV	HMPV	PIV1	PIV2	PIV3	RV	AdV	BoV	PIV4	CoV		EV	
													OC43	NL63		
2016/17	4	85	4	142	25	0	5	15	34	40	12	1	2	5	1	375
Outpatient	0	14	0	3	0	0	1	5	3	5	0	0	1	0	0	32
Hospitalized	4	71	4	139	25	0	4	10	31	35	12	1	1	5	1	343

All 398 patients were tested for RSV, HMPV, PIV type 1/2/3, RV, AdV, BoV using Real time PCR method. RSV accounted for 50,3% (142/282) of detected non-influenza viruses. The number and proportion (%) of detected human metapneumovirus, adenoviruses, rhinoviruses, parainfluenza viruses type 1, 2 and 3 among the identified non-influenza viruses were as follow: 25 (8.8%), 40 (14.1%), 34 (12%), (0 %), 5 (1.8%), 15 (5.3%), respectively. Human adenovirus was the second most common detected non-influenza virus (Figure 1).

A total of 93 samples negative for influenza and other respiratory viruses were tested also for HCoV (NL63 / OC43; 229E/HKU), EV, PIV4 by Multiplex PCR method. The results indicated coronaviruses NL63 in 5 (1.8%) and OC43 in 2 (0.7%) of the probes Multiplex PCR analyses confirmed also enterovirus in 1 (0.4%) of detected non-influenza viruses (Figure 1).

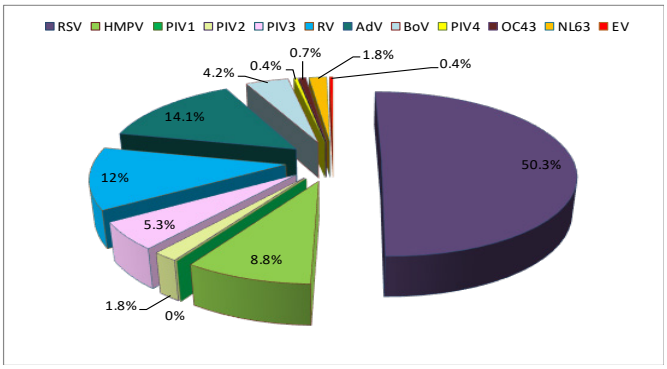


Fig. 1. Distribution (%) of proved non-influenza viruses in the 2016/2017 season in Bulgaria.

Overall, the most commonly determined pathogen among young children was RSV, followed by influenza A (H3N2) and AdV in single infections. Co-infections was detected in 53 (15.9%) patients and the here obtain combinations were as follows: RSV with A (H3N2) were identified in 13 samples, RSV with AdV in 10 of the cases, RSV with BoV in 9, RSV with HRV in 7, HMPV with AdV in 4, RSV with HMPV in 2, PIV2 with A (H3N2) in 1, PIV3 + with A (H3N2) in 1, PIV3 with HRV in 1, PIV3 with HRV in 1, RSV with Inf. B in 1, HRV with AdV in 1, BoV with AdV in 1, HRV with BoV in 1 patient respectively (Figure 2).

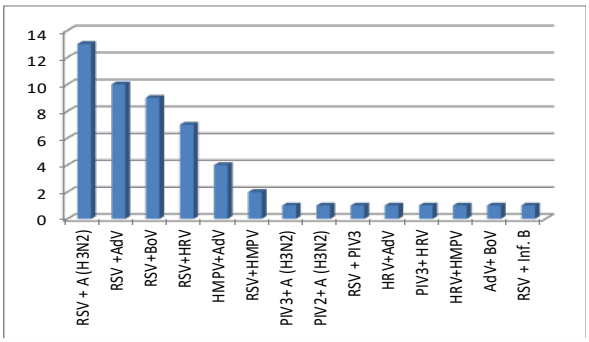


Fig. 2. Distribution (number) of proven co-infections with two respiratory viruses during the 2016/2017 season in Bulgaria among young children.

Clinical characteristics

During early childhood, respiratory viruses may cause serious complications to the lower respiratory tract (laryngotracheitis, bronchiolitis, pneumonia) or CNS (febrile seizures, brain edema, meningitis, encephalopathy, encephalitis). The contribution of influenza viruses, RSV, HMPV, HPIV1/2/3, rhinoviruses, adenoviruses, bocaviruses and HCoV in the development of complications mentioned above was analyzed. Figure 3 represent proportion (%) of patients infected with respiratory viruses in the groups with different clinical diagnosis (Figure 3).

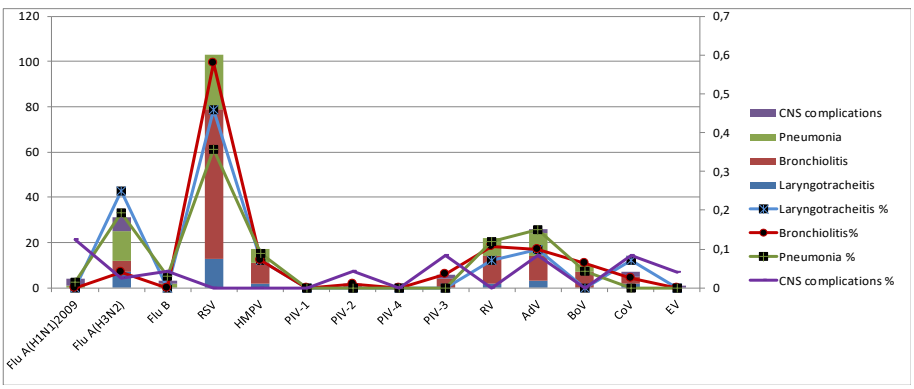
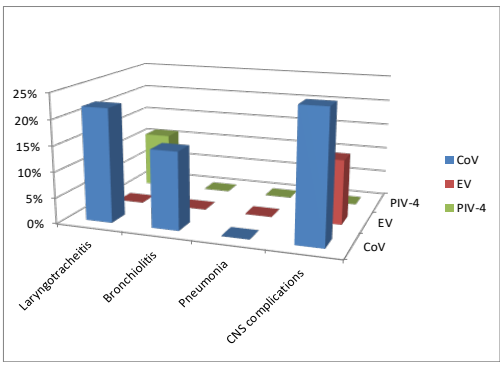


Fig. 3. Number and proportion (%) of patients with different clinical diagnosis and detected respiratory viruses

According to data on the figure 3 influenza viruses were the most common identified viruses among patients with neurological complications, laryngotracheitis and pneumonia accounting for 42%, 25% and 24% of the cases (Figure 3). Among patients with bronchiolitis, laryngotracheitis and pneumonia, RSV was the major pathogen and was found in 58%, 46% and 34% of samples, respectively. Coronaviruses and enteroviruses were found using Multiplex PCR in patients with neurological complications and the detection rate was 25% for CoV and 12.5% for EV (Figure 4).



Fig, 4. Proportion (%) of patients with different clinical diagnosis detected with CoV, EV and PIV4 using Multiplex PCR.

Among the patients with laryngotracheitis, CoV and PIV4 were detected in 22% and 11% of cases (Figure 4).

Respiratory tract infections are caused clinically by a multitude of pathogens. Pathogen-specific clinical symptoms are often lacking. The present study evaluated the clinical parameters in relations with virological status of medically attended ARTI among children under the age of 5 in Bulgaria during 2016/2017 winter season. Incidence of twelve respiratory viral infections were determined and their participation in the development of lower respiratory tract diseases and CNS complications are analyzed. Two molecular methods - Multiplex PCR and Real time PCR are used to screen the target spectrum of respiratory viruses. Viral etiology was demonstrated in 82% of the study cases. This incidence rate is higher than rates reported in some other studies (36.6% - 60.6%) (Zhang et al., 2014; Shafik et al., 2012; Liu et al., 2014). Co-infections (with two and three viruses) in this study were identified in a relatively low percentage of the tested children in Bulgaria (14%). According to various studies performed mainly using multiplex PCR, co-infections are common, occurring in 5 - 70% of ARI cases (Debiaggi et al., 2012; Cilla et al., 2008; Goka et al., 2014). There is no consensus on the impact of coinfection on disease severity. Some researchers suggest that infections with two or more viruses have been associated with more severe clinical symptoms, particularly co-infections involving RSV (Tregoning, and Schwarze, 2010; Tregoning et al., 2010).

Influenza epidemics are characterized by variations in the participation of the different types/subtypes of influenza viruses, their virulence and clinical activity. In Bulgaria, during the 2016/2017 winter season, predominant circulating influenza virus was A(H3N2). Influenza subtype A(H3N2) was identified in 85 (23%) of the tested samples, basically in cases with complications as bronchiolitis – 5 cases, pneumonia – 16 and with neurological complications - 10.

Respiratory syncytial virus is well known as the most important pathogen in relation to lower respiratory tract infections among infants and young children and the major causative agent of bronchiolitis and pneumonia (Hall, 2001). According to previous studies, RSV is responsible for 50-90% of hospitalizations with bronchiolitis and 5-40% of those with pneumonia among children under 5 years of age 4 (Eric et al., 2006; Hall, 2001). RSV was the most common identified pathogen in present research, accounting for 39% of all viruses detected. The frequency of RSV was higher among hospitalized children with complications to the lower respiratory tract, mainly bronchiolatis, laryngorheitis and pneumonia.

Another important causative agent of bronchiolitis and pneumonia like RSV in early childhood is BoV. Several authors indicate that monoinfections with HBoV are with relatively low frequency (17%), but co-infections with the involvement of BoV have high incidence rate (83%). Interestingly, co-infections involving RSV and BoV are common- up to 90% (Völz et al., 2007; Jiang et al., 2016). Our data also show a high frequency of co-infections including RSV and BoV – 9 of studied children were positive for these respiratory agents.

Rhinoviruses (type A, B and C) are the most common upper respiratory tract parhogens among patients of all ages. In young children, they can cause severe acute respiratory diseases, including middle otitis, sinusitis, croup, lower

respiratory tract infection (Kieninger et al, 2013). The present study indicated a relatively low frequency (8,5%) of rhinovirus infections. Rhinoviruses were found both in outpatients and in hospitalized children. Co-infections involving HRV, RSV and A(H3N2) were the most common according to the here represent data.

Human metapneumovirus also cause bronchiolitis and pneumonia in early childhood (Edwards et al., 2013). Our study identified 25 children positive for HMPV but only one of them was hospitalized for bronchiolitis.

The incidence of adenoviral infections varies between different pediatric studies. Adenoviruses cause more severe respiratory diseases mainly in immunocompromised patients. Forty children predominantly diagnosed with pneumonia were found positive for AdV according to our data.

Parainfluenza viruses type 1, 2, 3 and 4 show different clinical and epidemiological characteristics. Type -1 and -2 are important etiology agents of croup, whereas type 3 is mainly associated with the development of bronchiolitis and pneumonia in infants. In this study, parainfluenza virus types 1, 2 and 3 were found in a total of 20 (5%) children, mainly in these without complications (Mao et al., 2012).

Enteroviruses also cause respiratory illnesses in young children (Waghmare et al., 2015) Using Multiplex PCR method we determined only one enterovirus in patients with meningitis.

Four different human pathogenic coronavirus species known to date - 229E, OC43, NL63, HKU1 are associated with a range of respiratory symptoms, including high morbidity outcomes such as pneumonia and bronchiolitis (Gaunt et al., 2010). Although coronaviruses are globally distributed, there are temporally and geographic differences in the frequency of detection of the four viruses (Gaunt et al., 2010). Multiplex-PCR method can differentiate between 229E, OC43, NL63, and HKU1 (Krause et al., 2014). The use of a multiplex polymerase chain reaction to detect coronaviruses in respiratory sample is potentially beneficial, as it might help physicians to avoid unnecessary administration of antibiotics and overcomes diagnostic problems arising through seasonal variation in coronavirus frequency. Such approach also provides some novel data on clinical and epidemiology implications of coronaviruses. The here presented data indicate 7.7% detection rate in relation with coronaviruses among fully negative specimens of young children.

Predominantly, patients diagnosed with respiratory infections are positive for some of respiratory viruses mainly RSV, metapneumovirus, parainfluenza viruses and bocaviruses while among healthy children there are found a relatively high percentage of rhinovirus, coronavirus and enteroviruses. Possible explanation might be associated with prolonged exposure of the second group of viruses (Rhedin et al., 2014).

This study, basically carried out using the Real Time RT-PCR method, allows each clinical sample to be tested within 24 hours for the presence of the most common respiratory viruses. While, for detection of uncommonly proven viruses associated with respiratory syndromes, Multiplex PCR method was used. Unification of these two methods Real Time RT-PCR/ Multiplex PCR allows the

detection of a wide spectrum of viruses, etiological agents of acute respiratory diseases in pediatrics.

In conclusion, this study demonstrates the involvement of a wide range of viruses in the development of respiratory diseases during the 2016/2017 winter season in Bulgaria in infants and young children up to 5 years of age. The here obtain results include Real Time RT-PCR and Multiplex PCR analysis and confirm the leading role of RSV and influenza viruses as major etiologic agents of serious illnesses with the involvement of low respiratory tract and CNS in early childhood.

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