Original Article

Contribution of influenza viruses, human metapneumovirus and respiratory syncytial virus to acute respiratory infections in children in northern Greece, 2008 – 2010

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Abstract. Influenza viruses, respiratory syncytial virus (RSV) and human metapneumovirus (hMPV) are the most common pathogens that cause acute respiratory disease in children. The aim of this study is to present the contribution of the above three pathogens to influenza-like illness (ILI) in children (aged <6 years old) during 2-year (2008-2010) influenza seasons in N. Greece. 430 pharyngeal swabs from children younger than 6 years, presented as ILI infections during the last two influenza seasons (2008-2009 and 2009-2010) were examined for influenza A and B, RSV and hMPV, by one step Real-time RT-PCR. Influenza viruses were detected in 122 (28.3%) of the 430 spesimens, RSV in 45 (10.4%) samples and hMPV in 28 (6.5%). RSV and influenza viruses' co-infections were observed in eight cases, RSV and hMPV co-infections in four cases and hMPV with influenza viruses was found in one case. The majority of the patients (67.7%) were between 3 and 6 years old. Our results demonstrate that influenza viruses, RSV and hMPV contribute to ILI presenting infections at a rate of 45.2% in children younger than 6 years old.

Key words: influenza virus, human metapneumovirus (hMPV), respiratory syncytial virus (RSV), Northern Greece

1. Introduction

According to the World Health Organization (WHO), acute respiratory infections constitute the second most common cause of death in children younger than 5 years old globally. A variety of viruses, such as respiratory syncytial virus (RSV), human metapneumovirus (hMPV), parainfluenza and influenza viruses are responsible for the majority of respiratory disease cases in the pediatric population (1).

Influenza viruses A, B are leading causes of respiratory diseases in children as well as a significant number of hospitalizations among infants and children. Influenza season in Greece starts late in December and lasts until early April, peaking during January and February. Influenza attracted great medical interest recently due to the 2009 H1N1 Influenza pandemic. Beginning in April 2009, the novel influenza virus A (H1N1) spread across the world and two months later WHO declared a global pandemic. More than 213 countries and territories reported laboratory-confirmed cases of pandemic (H1N1) 2009 with more than 16.813 deaths as of March 21, 2010 (2). For this reason, the surveillance programme for the 2009-2010 season started in May 2009, instead of December 2009, in Greece.

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In 2001, van den Hoogen et al isolated a previously unknown viral pathogen from children in Netherlands. The virus was classified as a new member of the genus of Metapneumovirus and assigned the provisional name of hMPV, which is considered to be the cause of a significant proportion of both upper and lower respiratory infections in infants, children and adults as well (3).

Respiratory syncytial virus (RSV) on the other hand, is the main cause of lower respiratory track infections among children younger than 3 years old especially infants. Although RSV causes milder respiratory infections in older children, it causes bronchiolitis and pneumonia in infants with a mortality rate of 2 to 5 % (4). RSV is the most common cause of childhood acute lower respiratory infections (ALRI) globally and a major cause of admission to hospital. Moreover, mortality data demonstrate that RSV is a significant cause of death in childhood from ALRI, following pneumococcal pneumonia and Haemophilus influenza type b (5).

The National Influenza Centre for Northern Greece which is located in the 2nd Microbiology Department of Medical School, Aristotle University of Thessaloniki, one of two National Influenza Centers in Greece, examines clinical samples from patients with influenza-like illness (ILI) and other respiratory diseases, including infants and children, every year. The sentinel surveillance system, in which general practitioners and pediatricians are participating, is responsible for ILI surveillance in Greece since December 1999. Exceptionally, during the A (H1N1) 2009 influenza pandemic a significant number of specimens were collected from hospitalized patients who developed complications following the initial infection. Results are reported to the Hellenic Centre for Diseases Control and Prevention (HCIDC).

The aim of the present study is to determine the contribution of hMPV, RSV and influenza virus infections to ILI and other respiratory diseases, during the last two seasons 2008-2009 and 2009-2010, in northern Greece.

2. Materials and methods

During the period 2008-2010, pharyngeal swabs and/or washes were collected from a total of 430 patients (218 males and 212 females) under 6 years old in northern Greece, who were identified by practitioners as patients with ILI or other respiratory infection. The samples were sent to the National Influenza Centre for Northern Greece. National Influenza Centre for Northern Greece serves the northern part of the country and its population is 2.5 million people. All specimens were collected within three days from the onset of symptoms and they were transported to the laboratory in sucrose phosphate medium at the temperature of 4^{o} C within 24 hours. Moreover, every specimen was accompanied by a Standard Form, presenting the necessary demographic data, the date of specimen collection, clinical symptomatology and medical history of every case.

RNA extractions were performed using the Qiagen Viral R A mini kit according to manufacturer's instructions. The existence of influenza virus was checked by one step real-time RT-PCR with specific primers and probes for matrix protein and nucleoprotein genes of influenza A and B viruses (6). Until 2009, specimens were tested for the presence of influenza A (subtypes H1 and H3) and influenza B by one-step multiplex real-time RT-PCR (6). Since the emergence of the novel H1 influenza virus in 2009, specific primers and probes were used for the *in vitro* qualitative detection and characterization of swine influenza viruses in respiratory specimens (7). A multiplex real-time PCR protocol was used during the whole period of the study in order to investigate the existence of hMPV and RSV in specimens collected from young children with ILI or any other respiratory infection (8).

All results were registered and kept as a record file for the National Influenza Centre for northern Greece whereas following the determined guidelines a copy was additionally sent to HCIDC.

3. Statistical analysis

Statistical analysis of the results was performed by SPSS (version 16.0, SPSS Inc., Chicago, Illinois). The differences among age groups and infection rates were estimated by means of descriptive statistics. The chi-square test was used to compare the infection rates of influenza - RSV, influenza - hMPV and RSV – hMPV infections.

4. Results

During the period 2008-2010, pharyngeal swabs and/or washes were collected from a total of 430 patients (218 males and 212 females) under 6 years old in northern Greece, who were identified by practitioners as patients with ILI or other respiratory infection. From these specimens, 332

Table 1. Detection of influenza viruses, RSV and hMPV infections and coinfections in children, North Greece, 2008-2010

Influenza season	ILI cases	Influenza positive (%)	RSV positive (%)	hMPV positive (%)	Influenza +RSV (%)	Influenza +hMPV (%)	RSV+hMPV (%)
2008-2009	98	25 (25,5%)	29 (29,6%)	3 (3,06%)	8 (8,1%)	1 (1,02%)	0
2009-2010	332	97 (29,2%)	16 (4,8%)	25 (7,5%)	0	0	4 (1,2%)
Total	430	122 (28,3%)	45 (10,4%)	28 (6,5%)	8 (1,8%)	1 (0,2%)	4 (0,93%)

Table 2. Age distribution of ILI cases and confirmed influenza virus, RSV and hMPV infections, North Greece, 2008-2010

			Positive (n)				
	ILI cases total	Influenza total			RSV+	RSV	hMPV
Age groups	2008-09/2009-10	2008-09/2009-10	RSV	hMPV	hMPV	+influenza	+influenza
	30	9	5	0		1	
0-6 months	6/24	1/8	3/2	0/0	0	1/0	0
7months-	119	23	20	4	4	4	
2 years	36/83	11/15	17/3	0/4	0/4	4/0	0
	281	90	20	24		3	1
3-6 years	56/225	13/74	9/11	3/21	0	3/0	1/0
	430	122	45	28	4	8	1
total	98/332	25/97	29/16	3/25	0/4	8/0	1/0

were collected during 2008-9 and 98 in the years 2009-10. The patients were further divided into three age groups (0-6 months, 7months-2 years, 3-6 years). Furthermore, the patients were divided according to gender.

The median age of the 430 patients (218 males and 212 females) was 3.2 years (2 months-6 years old). Most of the young patients had upper respiratory infections, while only 23 patients developed pneumonia. Influenza viruses were detected in 122 specimens (28.3%), RSV in 45 (10.4%) and hMPV in 28 specimens (6.5%) Co-infections of RSV and hMPV were observed in 4 cases, RSV and influenza viruses in 8 cases (1.8%) and hMPV with influenza viruses in one case (0.2%) (Table 1).

During 2008-2009 influenza season (98 cases, 48 males and 50 females), 25 of the specimens (25.5 %) were positive for influenza viruses. The predominant subtype was influenza A (H3) (17 of 25, 68%), while RSV was the dominant cause of infection in 29 cases (29.6%) and hMPV was identified in three cases (3%). Co-infections of influenza virus and RSV were observed in eight cases (8.1 %), while influenza virus and hMPV in one case. Co-infection with RSV and hMPV was not identified (Table 1).

During the next 2009-2010 influenza season (332 cases, 170 males and 162 females), 97 (29.2%) of the specimens were positive for influenza viruses and Influenza A (H1 pandemic) was the predominant subtype (94 out of 97 specimens, 97%). RSV was the causative agent of infections in 16 cases (4.8%) and hMPV was detected in 25 cases (7.5%). Co-infections of RSV and hMPV were identified in four cases (1.2%), while co-infection of influenza and RSV or hMPV was not observed (Table 1).

According to the age distribution, the majority (n= 281, 65.3%) of the patients were 3-6 years old. Only 30 out of 430 patients were younger than 6 months of age (6.9%). The incidence of influenza infection was higher in the age group of 3-6 years old, as 90 out of 122 influenza infected patients (73.7 %) belonged to this group. Additionally, 24 out of 28 hMPV infected

patients (85.7 %) belonged to the age group of 3-6 years old. In contrast, the incidence of RSV infection was the same for both age groups, 7 months-2 years and 3-6 years (Table 2).

Statistical analysis of the results demonstrated that there was no statistical significant difference between age groups and influenza and RSV infection in the 2008-2009 influenza season (p=0.165 and p=0.937 respectively), while a statistically significant difference was observed between age groups and hMPV infection during the same season (p=0.000). Children aged 3-6 years old had a higher possibility of being infected by hMPV than children aged less than 3 years of age.

Furthermore, during the pandemic season 2009-2010, a statistically significant difference between the age groups and influenza and hMPV infections was found (p=0.004 for influenza and p=0.000 for hMPV), which means that children aged 3-6 years old had a higher possibility of being infected by influenza virus or hMPV. Moreover, during that season, all young patients had a higher possibility of being infected by influenza virus than RSV or hMPV (p=0.008 and p=0.001 respectively), something that can be very easily explained by the fact that 2009-2010 season was the pandemic one.

The most common clinical findings in patients were fever, rhinorrhoea and wheeziness. From the 23 patients with pneumonia, only 2 of them were positive for influenza viruses, 5 were RSV positive, 8 were hMPV positive and 2 had been co-infected with RSV and hMPV.

5. Discussion

Respiratory virus infections are an important cause of hospitalization for children. The aim of this study was to determine the contribution of influenza viruses, RSV and hMPV to respiratory infections in children during the last two influenza seasons 2008-2009 and 2009-2010 in northern Greece. According to our results, influenza viruses contributed to ILI at a rate of 28.3 %, RSV was responsible for 10.4 % of the respiratory infections and hMPV was the causative agent of 6.5 % of them. The prevalence of influenza viruses and hMPV was higher in the 3-6 years old age group (73.7 % and 85.7 %, respectively), while RSV appeared at the same rates (44.4%) in both 7 months-2 years old and 3-6 years old age groups.

During the 2008-2009 influenza season, 25.5 % of ILI patients were positive for influenza viruses, 29.6% of them were RSV-positive and only 3% of the patients were positive for hMPV. The following season (2009-2010), the rate of influenza positive patients was 29.2 %, while the rates for RSV and hMPV-positive were 4.8 % and 7.5 %, respectively. Due to 2009 H1N1 influenza pandemic, the influenza rates in children were increased in contrast to RSV rates which showed a significant decrease the same period. In addition, during the 2009-2010 season the hMPV infection rates were increased with respect to 2008-2009 season. In a similar study conducted in northern Greece as well during 2005-2008, the rates of influenza and hMPV infection in children (0-5 years old) were 24.4 % and 12.7 % respectively (9). In the present study, co-infections by influenza and RSV were found in 8 cases in the 2008-09 period, while none was observed in 2009-2010 one, in which co-infections by RSV and hMPV were detected in 4 cases. No statistical evaluation can be performed as these conifections were diagnosed in only four cases.

It is remarkable the fact that during the pandemic period, in which influenza incidence was much higher, the rate of influenza+RSV or influenza+hMPV coinfections is zero. This finding does not agree with similar findings from the literature, but it can be explained by the fact that young patients in the present study developed mostly upper respiratory infections, in which RSV and hMPV do not play as important role as a pathogen, as influenza virus does (10). Additionally, during the pandemic period, A(H1N1) pandemic virus was the predominant causative agent, which may not be involved in as many coinfections as the other subtypes of influenza virus. This finding needs to be cleared in a study in the near future.

No statistically significant difference in clinical symptomatology between RSV-hMPV co-infected patients and simply RSV or hMPV infected ones was observed, although Foulongne et al have recently suggested that hMPV/RSV co-infection is frequent and could be more severe than a single hMPV or RSV infection (11).

Our findings demonstrate that patients with pneumonia are more likely to be infected by hMPV (8 of 22, 36.3%), than RSV (22.7%) and influenza (9%). These findings are consistent with a similar study in Israel, in which hMPV infection was more often associated with a diagnosis of pneumonia than RSV and influenza viruses (12,13). In contrast, a previous study in Kenya demonstrates that RSV is the predominant viral pathogen for infants and children with

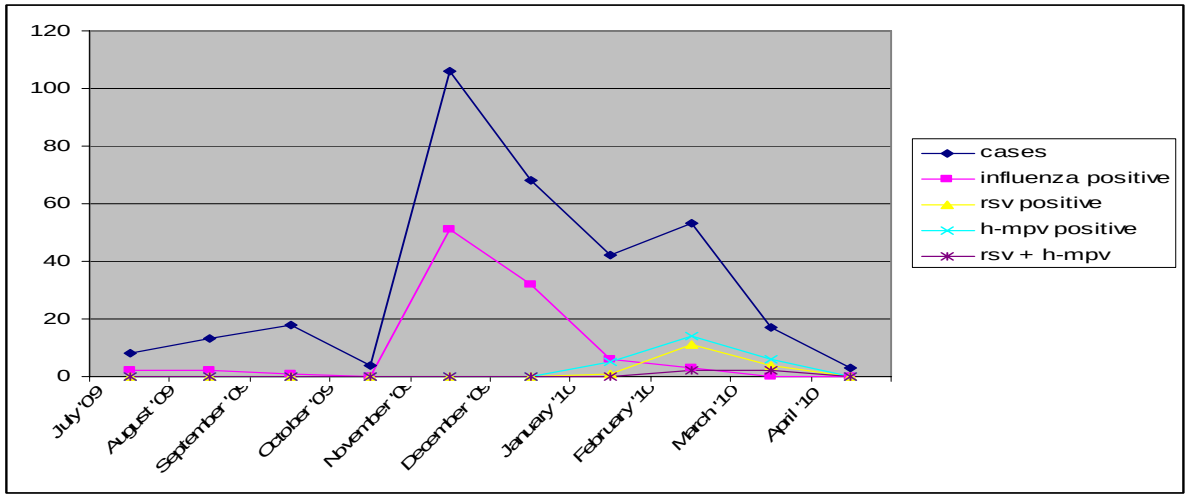

Fig. 1. Seasonal distribution of influenza virus, RSV and hMPV infections in children during influenza season 2009-10

severe pneumonia (14). This is probably due to the different geographical location of these countries.

In the influenza season 2008-2009, the hMPV as the RSV infection culminated in February-March and influenza infections reached a peak in December-January. Despite the fact that the surveillance programme for 2009-2010 season started in May 2009, instead of December 2009, the seasonal distribution of hMPV and RSV remained the same as the previous years. Most hMPV and RSV cases appeared in February while the majority of influenza cases were observed in November during that season (Figure 1). According to previous studies, the presence of hMPV and RSV infections are observed from the beginning to the end of each influenza season, peaking during winter-spring season (9,15,16).

A difference in viral agents (influenza+RSV+hMPV) detection rate in the two periods was also observed. 52 positive out of 98 examined cases (53%) in 2009-2009 and 138 positive out of 332 examined cases (42%) in 2009-2010. This can be explained by the fact that during the pandemic 2009-2010 influenza season, pediatricians and doctors in general were not familiar with the new pandemic influenza strain and its complications. This is the reason they sent respiratory specimens to the National Influenza Centre from young patients with mild symptoms, which turned to be negative.

In conclusion, our results show the significant role of the three pathogens – influenza viruses, hMPV and RSV – in acute respiratory disease in children. Further research is needed to elucidate the quantitative and qualitative importance of them, especially RSV and hMPV infections, their seasonal distribution, the groups at risk of severe complications and strategies for their diagnosis, treatment and prevention.

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