Research articles

APPLICATION OF RT-PCR FOR DIAGNOSIS OF RESPIRATORY SYNCYTIAL VIRUS AND HUMAN METAPNEUMOVIRUS INFECTIONS IN BULGARIA, 2006-7 AND 2007-8

S Pavlova (slavapavlova@ymail.com)¹, T Hadzhiova¹, P Abadjieva², R Kotseva¹
1. National Laboratory of Influenza and Acute Respiratory Diseases, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria
2. Second Multi-Profile Hospital for Active Treatment, Sofia, Bulgaria

We describe here the results of respiratory syncytial virus (RSV) detection by reverse transcription polymerase chain reaction (RT-PCR) during two consecutive seasons, from December 2006 to February 2007 and from October 2007 to March 2008, performed in the National Laboratory of Influenza and Acute Respiratory Diseases, Bulgaria. A total number of 278 nasopharyngeal samples obtained from hospitalised children up to the age of five years were investigated for these two seasons. During the first season, the aetiological role of RSV was confirmed in 56 of 148 samples (37.8%) compared to 11 of 130 samples (8.5%) during the second season. Since the beginning of January 2008, RT-PCR for the detection of the recently identified human metapneumovirus (HMPV) has also been introduced in Bulgaria. This virus has been demonstrated as the aetiological agent in 13 out of 81 samples (16%) from children of the same age group. The use of RT-PCR allows the detection of a broader spectrum of viruses causing respiratory diseases, as well as better discrimination of the aetiological agents in clinically similar cases.

Introduction

Acute respiratory infections (ARIs) represent a considerable health problem in infants and children. Despite the great number of viruses causing ARIs (more than 200), influenza viruses of type A and B, respiratory syncytial viruses (RSV), parainfluenza viruses, and adenoviruses are indicated traditionally among the most important aetiological agents of respiratory system diseases. However, in addition to previously known viruses, a number of respiratory viruses have been recently identified as causative agents of lower respiratory illnesses in children: human metapneumovirus

(HMPV), human coronavirus (HCoV-NL63), human bocavirus (HBoV) [1-3].

RSV is the major cause of bronchiolitis and pneumonia during the first years of life. Children with underlying illnesses such as congenital heart disease and bronchopulmonary dysplasia are at increased risk for severe infections due to RSV. In addition, RSV is increasingly recognised as an important pathogen in other groups, including immunocompromised patients and the elderly [4-6].

HMPV was first identified in the Netherlands in 2001 using a PCR designed for the identification of unknown agents multiplying in cell cultures [1]. Together with RSV, HMPV has quickly assumed an important position among the rest of the respiratory pathogens, particularly regarding early childhood diseases [7-9]. Clinical symptoms of HMPV infection seem to be indistinguishable from RSV infections. Major clinical manifestations of the infection caused by these two viruses in infants and young children are bronchiolitis and pneumonia [2,10,11].

Although routine diagnostic methods for respiratory viruses, including virus cultivation on cell culture, are robust, PCR for the detection of viruses in respiratory samples has also been shown to be useful because it offers an enhanced sensitivity combined with rapid detection [12,13]. In the past five years, RT-PCR has been applied as a highly sensitive and specific method for the diagnosis of RSV in the National Laboratory of Influenza and Acute Respiratory Diseases in Bulgaria. Since the beginning of 2008, RT-PCR has been used for HMPV detection in clinical samples as well [14-16].

TABLE 1
Number of positive initial specimens tested by different methods for RSV in children under five, Bulgaria, 2006-7 (n=148 samples tested)

Number of specimens tested	Isolation on cell cultures		RT-PCR- positive specimens
	Specimens positive for CPE	Specimens, confirmed by rapid tests	
148	24 (16.2%)	16 (10.8%)	56 (37.8%)

RSV: respiratory syncytial virus

The purpose of this study was to provide data about the detection of RSV and HMPV by RT-PCR in hospitalised children up to the age of five years during the period from 2006 to 2008 in Bulgaria.

Materials and methods

Clinical material

Nasopharyngeal samples were obtained by gently rubbing the deep nasal turbinate bilaterally with sterile swabs (Viral Culturette™ system, Becton Dickinson, Fisher Scientific), combined with a third swab from the posterior pharynx. Swabs were transported to the laboratory on the same day, dipped into a vial containing 2 ml saline and divided into aliquots. A fresh aliquot was used to inoculate a cell culture and the remaining aliquots were stored for PCR testing and further investigation. The samples were obtained from hospitalised children up to the age of five years admitted to a paediatric unit of the Second Multi-Profile Hospital for Active Treatment, Sofia, with signs and symptoms of an upper respiratory tract infection, bronchiolitis, or pneumonia. In the 2006-7 season, samples were also taken from Lozenetz Hospital and two orphanages in Sofia. During the two seasons investigated, approximately 1,900 children were covered by these hospitals and by the two orphanages. The following indicators were taken into consideration for children's hospitalisation: infants younger than five years and high risk infants (prematurely born infants, children

with bronchopulmonary dysplasia and congenital heart diseases, dystrophia, children born from twin pregnancies).

Cell culture

Samples (0.2 ml) were inoculated on the day of collection onto HEp-2 and MRC-5 cells. The cell cultures were moved to the Cell Cultures Laboratory, National Center of Infectious and Parasitic Diseases (NCIPD). Cultures were observed daily during 10 days for cytopathic effect (CPE). RSV produces a characteristic CPE consisting of syncytia formation. When the CPE had reached 50% or more of the monolayer, the culture supernatant was aspirated for subsequent virus identification. RSV infection was confirmed by enzyme immunoassay (EIA) membrane test for the rapid and qualitative detection of RSV (Directigen RSV, Becton Dickinson) [15].

RNA Extraction

RNA was extracted using Trizol LS reagent (Invitrogen) or RiboSorb (Sacace) kits, according to the manufacturer's instructions.

RT-PCR

RT-PCR for RSV detection was performed by ABGene iT-One Step RT-PCR (Invitrogen), using specific primers directed against a 278 nt fragment in the highly conservative region of the nucleocapsid gene of RSV (position 858-1135) [18].

RT-PCR for HMPV detection was performed by Qiagen® One Step RT-PCR Kit (Qiagen) kit using specific primers directed against a 416 nt fragment of the matrix protein gene of HMPV [17].

RNA extracted from the RSV reference strain, maintained through multiple passaging in a HEp-2 cell line, was used as a positive control for RSV. As positive control for HMPV, extracted RNA received from the Cantacuzino Institute, Romania was used [17]. As negative control distilled water was used.

Results

First season (2006-7)

A total of 148 samples from hospitalised children were tested by RT-PCR for RSV during this first period of study. Table 1 demonstrates that 56 (37.8%) samples were RSV-positive. In the parallel testing of all of the samples on cell cultures, 24 samples (16.2%) showed a CPE, expressed mainly in round-cell degeneration of the monolayer without the appearance of the syncytia characteristic for RSV-infected cells. In addition, the culture supernatants were tested by a rapid immuno-chromatographic test assay membrane test (Directigen RSV) as soon as the CPE had spread over at least 50% of the monolayer (between 48 h and 10 days post inoculation).

FIGURE

Distribution of samples positive for RSV and influenza viruses in children under five, Bulgaria, 2006-7 and 2007-8

RSV: respiratory syncytial virus

TABLE 2

Distribution by week of the RT-PCR-positive specimens for RSV in children under five, Bulgaria, 2006-7 (n=148 samples tested)

	December 2006 – February 2007							
	Week 49	Week 50-51	Week 52-1	Week 2	Week 3	Week 4	Week 5	Week 6
Number of specimens tested	12	12	5	38	26	28	10	17
Number of positive specimens (percent)	2 (16.6%)	3 (25%)	4 (80%)	32 (84.2%)	7 (26.9%)	5 (17.9%)	1 (10%)	2 (11.8%)

RSV: respiratory syncytial virus

This test was positive in 16 of the samples cultured (10.8% of all tested samples).

Laboratory-confirmed cases of RSV (Figure) showed a peak in January 2007 (48 RSV-positive results). A lower number of positive samples was detected in December 2006 and February 2007 (a total of eight positive samples).

In Table 2, the distribution of the positive specimens is indicated by weeks, which demonstrates that during the peak period, over 84% of samples were RSV-positive.

On the background of the advancing influenza epidemic in the beginning of 2007, RSV infections peaked almost two weeks earlier. It is worth noting that the RSV curve was based on testing of children under the age of five years, received from December 2006 to February 2007, while the influenza curve was based on all clinical specimens (n=732) received in the laboratory during the period from October 2006 to March 2007 in relation to the laboratory follow-up of the epidemic circulation of the seasonal influenza viruses. Nevertheless, the similar course of the two curves is evident, which confirms that both viruses – RSV and influenza A virus - contribute simultaneously to the morbidity rate attributable to influenza and other acute respiratory infections (Figure) [19,20]. The results obtained indicate an increase in RSV infections from the end of December 2006, and a decrease from mid-January 2007 (Table 2).

The age distribution of the 56 positive samples for RSV according to their clinical diagnosis is presented in Table 3. The

TABLE 3

Distribution of RSV-positive samples in children under five by age group and clinical diagnosis, Bulgaria, 2006-7 (n=56)

Clinical diagnosis	Age groups		
	0 - 12 months	1 - 3 years	4 - 5 years
	RSV	RSV	RSV
Acute bronchiolitis	20	7	0
Bronchitis or acute rhinopharyngitis	15	6	0
Pneumonia	2	3	3

RSV: respiratory syncytial virus

TABLE 4

Distribution by month of samples positive by RT-PCR for RSV and HMPV in children under five, Bulgaria, 2007-8 (n=130 samples tested)

	October 2007 – March 2008*					
	October 2007	November 2007	December 2007	January 2008	February 2008	March 2008
Number of samples tested for RSV	18	13	18	42	32	7
Number of samples tested for HMPV	0	0	0			
Number of positive samples	0	0	0	3 RSV 2 HMPV	8 RSV 8 HMPV	3 HMPV

*A total of 130 samples were tested for RSV, 81 of which were also tested for HMPV
RSV: respiratory syncytial virus; HMPV: human metapneumovirus

largest number of RSV positive samples was recovered from the youngest age group from 0 to 12 months (37 positive samples).

Second season (2007-8)

As a continuation of the work done in the first season, RT-PCR was applied as a routine method for confirmation of RSV directly to clinical materials. The successful application of RT-PCR for RSV diagnostics in the season 2006-7 encouraged us to include during the season 2007-8 diagnostics of the newly emerging pathogen HMPV.

A total of 130 nasopharyngeal swabs were tested for RSV in the period from October 2007 to March 2008 (Table 4). The clinical material originated from hospitalised children up to the age of five years. Eighty-one swabs were received after the beginning of 2008 and they were tested in parallel for both RSV and HMPV. Using RT-PCR, the presence of RSV RNA was confirmed in 11 samples (8.5%), and 13 samples (16.1%) were positive for HMPV.

The age distribution of positive RSV and HMPV cases is presented in Table 5. The largest number of HMPV positive samples was recovered from the youngest age group from 0 to 12 months (10 positive samples). RSV confirmation has been comparatively lower in all age groups for the second period than for the first.

Based on the number of laboratory-confirmed RSV cases found, RSV infections were limited to individual cases in the season 2007-8, in contrast to the 2006-7 season, when an RSV epidemic was observed (Figure). In contrast to the CPE caused by the RSV isolated in 2007, which resulted in round cell degeneration of the monolayer, the strains isolated in 2008 caused RSV-typical syncytia in the monolayer. It is possible that this was due to differences in the biological characteristics of the strains isolated in the two seasons, indicating that they may belong to different RSV strains. This will be an object for further investigations.

Superimposed on the RSV results in the Figure, there is a curve showing the number of clinical samples found positive for influenza during the period under discussion. While the peak of the RSV and influenza activity coincided in the season 2006-7, the season 2007-8 saw first a peak in influenza-positive samples and, after the influenza activity had decreased, a peak in RSV-positive samples. Nevertheless, morbidity due to acute respiratory infections during this second season remained constant, indicating that RSV and influenza contribute together to the overall morbidity [20].

As HMPV testing is new in laboratories in Bulgaria, it seems too early at this stage to draw any conclusions about the epidemic distribution of this virus.

Discussion and conclusion

The development of molecular techniques for diagnosis of respiratory pathogens that cannot be cultured easily by traditional techniques has revolutionised the field of virology and infectious diseases. Even if certain viruses such as RSV can be grown in cell culture, this method is not completely reliable and many scientists have begun to use RT-PCR to identify infection [21]. The diagnosis of HMPV infection is even more problematic, as the virus is difficult to isolate in cell culture [1,22]. RT-PCR examination of respiratory secretions is currently the clinical test chosen for reliable diagnosis of HMPV. This is a reason to start using molecular tests for diagnostics of important respiratory viral causative agents such as RSV and HMPV.

For RSV, we detected 37.8% positive samples by PCR versus 10.8% by cell culture in the first season, and 8.46% versus 1.5% in the second season. This is in accordance to the results of other authors who also report a higher percentage of RSV-positive samples detected by PCR than by cell culture [13]. Some scientists have also tried to use cell lines for the isolation of HMPV [1,23]. As HMPV isolation is difficult to achieve in a cell culture model (non-characteristic cytopathic effect and necessity of prolonged cultivation), RT-PCR remains the single alternative for laboratory confirmation of its aetiological role. In this study, we found 16% HMPV-positive samples by RT-PCR. The largest number of positive samples was recovered from the youngest age group. These data coincide with information published by other researchers [24]. However, due to the limited number of tested samples, it is too early to draw a final conclusion regarding the role of this virus in the ARIs morbidity in Bulgaria.

Keeping in mind the literature data according to which the pathogens described infect preferably the youngest age group, we have focused our diagnostic study on hospitalised children up to the age of five [4,25-26]. We believe that the high percentage of positive results by RT-PCR - more than 84% in the second week during the first season - is an indication that we chose the right age group of children to be tested. This is in accordance with the clinical diagnosis and the intensive circulation of the virus during this period (Table 2, Table 3) [19].

The laboratory confirmation of RSV and HMPV aetiology by RT-PCR gives a prompt response within 24 to 48 hours, which is important for the therapy and critical for effective patient

management by focusing appropriate drug treatment, reducing unnecessary use of antibiotics, and preventing nosocomial spread [13,27-29]. Especially because of the necessity of rapid result for the clinician, virus isolation on cell culture is being displaced by molecular biology tests. Nevertheless, the isolation of the viral causative agent remains a gold standard and a basic model for the study of genetic and antigenic changes in the virus population, as well as a means of detection of new respiratory viruses [30].

The clinical picture of HMPV (bronchiolitis or pneumonia among infants and young children) initially resembles the one caused by RSV [24]. A co-infection with both viruses is possible as well, being associated with more severe course of the disease [2,31]. In our study we have not detected such cases. According to data obtained from the second investigated period, there were no clinical criteria for distinguishing both viruses. The highest number of positive samples for both viral agents is in the youngest age group (0-12 months) and they are associated with severe course of the disease, which has also been observed by other authors [32].

In many European countries, investigations were performed of the incidence not only of influenza viruses, but also of RSV as an important pathogen with social and economical impact especially in early childhood [20,33,34]. Increasing circulation of RSV is registered in countries in the European Union: in England, 40.8% of RSV-positive sentinel samples are found in young children aged 0 to 4 years, whereas in Scotland and France this proportion is approximately 11% [20]. The same authors reported that about 92% or more of RSV-positive non-sentinel samples were obtained from 0 to 4 year-old children. In our investigation, we obtained 37.8% positive results for the same age group during the first season. That this percentage is lower than reported in the literature is probably due to limited sample collecting that covered only one hospital in that period. The percentage of laboratory-confirmed RSV cases in Greece for the same season was 5.4%, mainly among children up to the age of three years [35]. Taking into account the fact that the RSV distribution varies in different countries and seasons [34], we consider that the smaller confirmed number of positive results for RSV that we obtained in the season 2007-8 reflects the true situation in Europe.

The epidemic spread of influenza viruses in Bulgaria for the two observed epidemic seasons coincides with the epidemic incidence of influenza viruses in Europe, and influenza was the most commonly detected virus in all European countries [36]. Routine detection of other viral respiratory pathogens yields data which are useful in monitoring general trends in morbidity from ARIs. In current investigations the detection of RSV in clinical samples coincides also with epidemic spread of influenza viruses [37,38]. During the second season, the peak of RSV infections did not coincide with that for influenza virus infections [39].

Many investigators mark the importance of collecting circulation data not only for influenza viruses but also for other causative agents of ARIs, and RSV is one of the most important. It is really necessary to build an RSV surveillance in Europe, in order to broaden and represent the real rate and spectrum of viral respiratory diseases [20,40].

In conclusion, the present results give information about the spread of two respiratory viruses - RSV and HMPV - among hospitalised children, detected by RT-PCR in Bulgaria. Collecting information on the spread of RSV is a requirement from the European Influenza Surveillance Scheme (EISS), which underlines

TABLE 5

Distribution of RSV- and HMPV-positive samples in children under five by age group and clinical diagnosis, Bulgaria, 2007-8 (n=24)

Clinical diagnosis	Age groups					
	0 - 12 months		1 - 3 years		4 - 5 years	
	RSV	HMPV	RSV	HMPV	RSV	HMPV
Acute bronchiolitis	7	9	2	2	0	0
Pneumonia	2	1	0	0	0	1

RSV: respiratory syncytial virus; HMPV: human metapneumovirus

the necessity of collection of data regarding the incidence of this virus in different European countries. The investigations reported here are a priority for the National Laboratory of Influenza and Acute Respiratory Diseases in Bulgaria in order to confirm the participation of some more widely distributed viruses, as causative agents of ARIs, first and foremost in paediatric pathology.

Acknowledgements

We would like to thank our colleagues from the Laboratory of Influenza, Cantacuzino Institute, Bucharest, Romania, for providing us with primers for HMPV and RT-PCR protocol for the detection of this virus.

References

- Van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, et al. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *NatMed* 2001;7(6):719-24.
- Choi EH, Lee HJ, Kim SJ, Eun BV, Kim NH, Lee JA, et al. The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000-2005. *Clin Infect Dis* 2006;43(5):585-92.
- Ebihara T, Endo R, Ma X, Ishiguro N, Kikuta H. Detection of human coronavirus NL63 in young children with bronchiolitis. *J Med Virol* 75(3):463-5.
- Hall CB. Respiratory syncytial virus and parainfluenza virus. *N Engl J Med* 2001;344(25):1917-28.
- Collins PL, Chanock RB, Murphy BR. Respiratory syncytial virus. In B. N. Fields (ed.), *Virology*. Forth edition. Philadelphia: Lippincott Williams and Wilkins, Philadelphia. 2001;1443-85.
- Walsh EE, Falsey AR, Hennessy PA. Respiratory syncytial virus and other infections in persons with chronic cardiopulmonary disease. *Am J Respir Crit Care Med* 1999;160(3):791-5.
- Dollner H, Risnes K, Radtke A, Nordbo SA. Outbreak of human metapneumovirus infection in norwegian children. *Pediatr Infect Dis J* 2004;23(5):436-40.
- Van den Hoogen BG, van Doornum GJ, Fockens JC, Cornelissen JJ, Beyer WE, de Groot R, et al. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. *J Infect Dis* 2003;188(10):1571-7.
- McIntosh K, McAdam AJ. Human metapneumovirus-an important new respiratory virus. *N Engl J Med* 2004;350(5): 431-3.
- Falsey AR, Erdman D, Anderson LJ, Walsh EE. Human metapneumovirus infections in young and elderly adults. *J Infect Dis* 2003;187(5):785-90.
- Wolf DG, Greenberg D, Kalkstein D, Shemer-Avni Y, Givon-Lavi N, Saleh N, et al. Comparison of human metapneumovirus, respiratory syncytial virus and influenza A virus lower respiratory tract infections in hospitalized young children. *Pediatr Infect Dis J* 2006;25(4):320-4.
- Stockton J, Ellis JS, Saville M, Clewley JP, Zambon MC. Multiplex PCR for typing and subtyping influenza and respiratory syncytial viruses. *J Clin Microbiol* 1998;36(10): 2990-5.
- Falsey AR, Formica MA, Walsh EE. Diagnosis of respiratory syncytial virus infection: comparison of reverse transcription PCR to viral culture and serology in adults with respiratory illness. *J Clin Microbiol* 2002;40(3):817-20.
- Hadzhiolova T, Kotseva R, Kalvachev Z, Dundarova D, Mladenova Z. Application of a complex of laboratory methods for identification of Influenza viruses and RSV in participation of Laboratory of Influenza and ARD in Second European Qualitative Control. *Infectology* 2004; XLI: 21-26.
- Hadzhiolova T, Pavlova S, Kotseva R. Possibilities for laboratory diagnosis of Respiratory Syncytial Virus. *Biotechnology* 2005;2(19):67-7111.
- Pavlova S, Hadzhiolova T, Kotseva R, Abadgieva P. Application of RT-PCR for detection of RSV in hospitalized children. *Infectology* 2007;1:119-22.
- ecu C, Orasanu D, Sima A, Mihai M, Alexandrescu V, Matei D, et al.: First detection of human metapneumovirus in children with respiratory infections in Romania. *Roum Arch Microbiol Immunol* 2007;66(1-2):37-40.
- Valdivia A, Savón C, Chacón D, Sarmiento L, Morier L, Otero A, et al. Analysis of respiratory syncytial virus in clinical samples by reverse transcriptase-polymerase chain reaction restriction mapping. *Mem Inst Oswaldo Cruz* 1997;92(3):389-93.
- Meury S, Zeller S, Heininger U. Comparison of clinical characteristics of influenza and respiratory syncytial virus infection in hospitalised children and adolescents. *Eur J Pediatr* 2004;163(7):359-63.
- Meerhoff TJ, Fleming D, Smith A, Mosnier A, van Gageldonk-Lafeber AB, Paget WJ, et al. Surveillance recommendations based on an exploratory analysis of respiratory syncytial virus reports derived from the European Influenza Surveillance System. *BMC Infect Dis* 2006;6:128.
- Zambon MC, Stockton JD, Clewley JP, Fleming DM. Contribution of influenza and respiratory syncytial virus to community cases of influenza-like illness: an observational study. *Lancet* 2001;358(9291):1410-6.
- Van den Hoogen BG, Osterhaus DM, Fouchier RA. Clinical impact and diagnosis of human metapneumovirus infection. *Pediatr Infect Dis J* 2004;23(1 Suppl): S25-32.
- Abiko C, Mizuta K, Itagaki T, Katsushima N, Ito S, Matsuzaki Y, et al. Outbreak of human metapneumovirus detected by use of the Vero E6 cell line in isolates collected in Yamagata, Japan, in 2004 and 2005. *J Clin Microbiol* 2007;45(6):1912-9.
- García-García ML, Calvo C, Martín F, et al. Human metapneumovirus infections in hospitalized infants in Spain. *Archives of Disease in Childhood* 2006; 91:290-295
- Selwyn BJ. The epidemiology of acute respiratory tract infection in young children: comparison of findings from several developing countries. Coordinated Data Group of BOSTID Researchers. *Rev Infect Dis* 1990;12 Suppl 8:870-88
- García-García ML, Calvo C, Perez-Brena P, De Cea JM, Acosta B, Casas I. Prevalence and clinical characteristics of human metapneumovirus infections in hospitalized infants in Spain. *Pediatr Pulmonol* 2006;41(9):863-71.
- Wihley DM, Symmis MW, Mackay IM, Sloots TP. Detection of human respiratory syncytial virus in respiratory samples by LightCycler reverse transcriptase PCR. *J Clin Microbiol* 2002;40(12): 4418-22.
- Groothuis JR, Wooden KA, Katz R, Robertson AD, McBride JT, Hall CB, et al. Early ribavirin treatment of respiratory syncytial viral infection in high-risk children. *J. Pediatr* 1990;117(5):792-8.
- O'Callaghan CA. Prevention of nosocomial respiratory syncytial virus infection. *Lancet* 1993;341(8838):182.
- Nunes IF, Moura FE. Isolation of respiratory syncytial virus from nasopharyngeal aspirates stored at 20 degrees C from one to fifteen months after collection. *Mem Inst Oswaldo Cruz* 2006;101(4):451-3.
- Greensill J, McNamara PS, Dove W, Flanagan B, Hart CA. Human metapneumovirus in severe respiratory syncytial virus bronchiolitis. *Emerg Infect Dis* 2003;9(3):372-5.
- Ginocchio CC, Manji R, Lotlikar M, Zhang F. Clinical evaluation of the NucliSENS analyte-specific reagents for the detection of human metapneumovirus in pediatric respiratory specimens. *J Clin Microbiol* 2008;46(4):1274-80. Epub 2008 Feb 13.
- Domegan L, O'Donnell J, Cunney R, O'Kelly E, Dooley S. Increased respiratory syncytial virus activity in Ireland. *Euro Surveill* 2002;6(49):pii=1980. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=1980>
- Terletskaia-Ladwig E, Enders G, Schallasta G, Enders M. Defining the timing of respiratory syncytial virus (RSV) outbreaks: an epidemiological study. *BMC Infect Dis* 2005;5(1):20.
- Gioula G, Melidou A, Exindari M, Chatzidimitriou D, Kyriazopoulou-Dalaina V. Contribution of RSV and hMPV to patients with influenza-like infections during 2006-2007 season in northern Greece, 2008; Abstract. 18th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain
- Arkema JM, Meijer A, Meerhoff TJ, Van Der Velden J, Paget WJ, European Influenza Surveillance Scheme EISS). Epidemiological and virological assessment of influenza activity in Europe, during the 2006-2007 winter. *Euro Surveill* 2008;13(34):pii=18958. Available from: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=18958>
- Kojouharova M, Kurchatova A, Vladimirova N, et al. News Bulletin of the National Center of Infectious and Parasitic Diseases (NCIPD) [in Bulgarian]. 2008;6:4-40.
- Izurrieta HS, Thomson WW, Kramarz P, Shay DK, Davis RL, DeStefano F, et al. Influenza and the rates of hospitalization for respiratory disease among infants and young children. *N Engl J Med* 2000;342(4):232-9.
- Gasparini R, Ansaldi F, Banfi F, Ferro G, et al. The impact of influenza and respiratory syncytial virus in a 0-14-year children cohort: a comparison with the national network and between methods for influenza virological surveillance. *International Congress Series* 2004; 329-33.
- Meerhoff TJ, Paget WJ, Aguilera JF, van der Velden J. Harmonising the virological surveillance of influenza in Europe: results of an 18-country survey. *Virus Res* 2004;103(1-2):31-3.

This article was published on 11 June 2009.

Citation style for this article: Pavlova S, Hadzhiolova T, Abadgieva P, Kotseva R. Application of RT-PCR for diagnosis of respiratory syncytial virus and human metapneumovirus infections in Bulgaria, 2006-7 and 2007-8. *Euro Surveill* 2009;14(23):pii=19233. Available online: <http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19233>