ISSN 1314-6246 Georgieva et al. 2016 *J. BioSci. Biotechnol.* ***2017***, SE/ONLINE: 69-75
RESEARCH ARTICLE

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Respiratory syncytial virus infection among children aged under 5 years in Bulgaria during two consecutive winter seasons 2014-15 and 2015-16

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ABSTRACT

Human respiratory syncytial virus (HRSV) is the most common viral etiological agent of acute respiratory tract infections, moderate and severe respiratory diseases like bronchiolitis and pneumonia in infants. It is a major cause for hospitalization during infancy and early childhood.

This study aims to provide data about circulation and molecular characteristics of HRSV detected in Bulgarian children less than 5 years of age with acute respiratory illness (ARI) or influenza-like illness (ILI) during two consecutive winter seasons – 2014/15 and 2015/16.

A total of 578 nasopharyngeal swabs of children, aged < 5 years with ARTI or ILI were tested using Real Time RT-PCR reactions based on specific primers/probe. Phylogenetic analysis of nucleotide and amino acid sequences were done for 46 of HRSV positive samples.

Respiratory-syncytial virus was confirmed in 154 (26.64%) samples, of which 55 (41.98%) were classified as HRSV-A and 71 (54.2%) as HRSV-B. HRSV was detected in 31 (50.8%) of cases of bronchiolitis and 30 (40%) of pneumonia. All analyzed HRSV-A strains belonged to the new ON1 genotype. HRSV-B strains belonged to BA9 and BA10 gene groups. Epidemiological and clinical features of HRSV infections are also analyzed.

Conclusion: The study highlights the significance of HRSV as a major etiologic agent in pediatrics and need for continued virological and epidemiological surveillance.

Key words: Human respiratory syncytial virus, molecular characteristics, bronchiolitis, pneumonia, children

Introduction

Infections of the upper and lower respiratory tract are very common in early childhood. One of the most important etiological agents of such infections and leading cause of hospitalization of children <5 years of age, is human respiratory syncytial virus (HRSV) (C. Hall 2012). Severe lower respiratory syndromes associated with HRSV infection include pneumonia and bronchiolitis. Pneumonia is the leading cause of childhood mortality among young in all regions of the world (Bryce, et al. 2005, Nair, et al. 2010).

Human respiratory syncytial virus causes annual epidemics in winter months that coincide with the flu season. Based on genetic and antigenic variations in structural proteins, HRSV isolates have been subdivided into two major antigenic groups (A and B). Both subgroups are associated with different severities of infection (Gilca, et al. 2006, Mentel, et al. 2005). There are several known molecular technics for detection of HRSV (Stockton, et al. 1998, Pavlova, et al. 2007). Since 2014, real-time RT-PCR has been applied in the National Reference Laboratory of Influenza and ARD, as routine diagnostic method for the detection of HRSV in samples of Bulgarian children <5 years of age. The aim of this study was to provide data about circulation and molecular characteristics of HRSV detected in Bulgarian children less than 5 years of age with acute respiratory illness (ARI) or influenza-like illness (ILI).

Materials and Methods

A total of 578 nasopharyngeal swabs of children, aged < 5 years were enrolled in the present study. The patients were ambulatory treated or hospitalized for influenza like illness

(ILI) or acute respiratory illness (ARI). The clinical diagnoses were reported by the physicians who provided the specimens. The samples were received in the National Reference Laboratory of Influenza and ARD, from different regions of the country during two consecutive winter seasons - 2014-15 and 2015-16, according to National Influenza Pandemic Preparedness Plan. This study was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans http://www.healthscience.net/resources/declaration-of-helsinki/ and approved by the local Ethics Committee of the NCIPD

Extraction of nucleic acids

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.

Real time RT-PCR

Detection of HRSV viruses was carried out by Real Time RT-PCR reactions based on specific primers/probe with the use of a kit SuperScript® III Platium® One Step Quantitative RT-PCR System. The sequences of the primer set and the 6-carboxyfluorescein (FAM)-labeled probe were summarized in Table 1. The following cycling conditions were used: reverse transcription at 45°C for 10 min, Taq inhibitor inactivation at 94°C for 10 min, and 45 cycles of denaturation at 94°C for 30 s followed by annealing/amplification at 60°C for 1 min.

Multiplex qRT-PCR assay

Subtyping was performed for HRSV-positive samples by multiplex qRT-PCR assay. HRSV-A-specific primer set and probe containing a 5´ reporter dye, 6-carboxyfluorescein, were directed to the F gene, while an HRSV-B-specific primer set and probe containing the 5´ reporter dye VIC were directed to the N gene (Table 1). Real-time RT-PCR was carried out under the following conditions: 48°C for 30 min, 95°C for 10 min and 45 cycles of amplification (15 s at 95°C and 1 min at 60°C).

Nucleotide sequencing

RSV-A and RSV-B specific oligonucleotide primers were used for sequencing of the G gene as summarized in Table 1. The reverse transcription (RT)-PCR assay was performed with a One Step RT-PCR kit (Qiagen) using following protocol: 55°C for 30 min; 94°C for 16 min; 40 cycles of 94°C for 30 s, 63°C for 1 min (58°C for 30 sec for HRSV-A), 72°C for 1 min, and final extension step at 72°C for 10 min. All the amplified products were subjected to 1.5% agarose gel electrophoresis.

Table 1. *HRSV-A and -B primers and probes.*

Primer/Probe	Gene	Sequence 5'→3'	Reference
Real time RT-PCR			
RSV-F	M	GGC-AAA-TAT-GGA-AAC-ATA-CGT-GAA	(Kodani, et al. 2011)
RSV–R	M	TCT-TTT-TCT-AGG-ACA-TTG-TAY-TGA-ACA-G	(Kodani, et al. 2011)
RSV-Probe		FAM-CTG-TGT-ATG-TGG-AGC-CTT-CGT-GAA-GCT	(Kodani, et al. 2011)
Multiplex qRT-PCR			
HRSV-A – AFF	F	CTGTGATAGARTTCCAACAAAAGAACA	(Zlateva, et al. 2007)
HRSV-A – AFR	F	AGTTACACCTGCATTAACACTAAATTCC	(Zlateva, et al. 2007)
HRSV-A F-TP – Probe	F	FAM-CAGACTACTAGAGATTACC	(Zlateva, et al. 2007)
HRSV-B – BNF	N	GGCTCCAGAATATAGGCATGATTC	(Zlateva, et al. 2007)
HRSV-B – BNR	N	TGGTTATTACAAGAGCAGCTATACACAGT	(Zlateva, et al. 2007)
HRSV-B N-TP – Probe	N	VIC-TATCATCCCACAGTCTG	(Zlateva, et al. 2007)
Nucleotide sequencing			
RSVA-G513-F	G	AGTGTTCAACTTTGTACCCTGC	(Aamir, et al. 2013)
RSVA-F131-R	G	CTGCACTGCATGTTGATTGAT	(Aamir, et al. 2013)
RSVB - Forward primer BGF	G	GCAGCCATAATATTCATCATCTCT	(Aamir, et al. 2013)
RSVB - Reverse primer BGR	G	TGCCCCAGRTTTAATTTCGTTC	(Aamir, et al. 2013)

The amplicons were purified using a PureLink® Quick Gel Extraction Kit (Invitrogen), according to the manufacturer's instructions. The purified PCR products were sequenced in the forward and the reverse directions on a GenomeLab GeXP Genetic Analysis System by using GenomeLab™ Dye Terminator Cycle Sequencing Quick Start Kit.

Phylogenetic Analysis

The nucleotide sequences were aligned with the HRSV sequences retrieved from the GenBank database. Phylogenetic trees were generated using the Maximum Likelihood method with MEGA, version 6 (Tamura, et al. 2013). Statistical significance of the tree topology was tested by bootstrapping in 1,000 pseudoreplicates. For both RSV groups A and B, phylogenetic trees were constructed by using reference sequences from the C-terminal variable region of the gene encoding G protein of representative strains from all known genotypes.

Results

Clinical and virologic characteristics

The present study included two consecutive winter epidemic seasons in Bulgaria: from October 2014 to May 2015 and from October 2015 to May 2016. The study population consisted of 578 children aged < 5 years presenting with ILI or ARI. During the 2014/2015 season 196 children were tested, during the 2015/2016 season – 382; 315 (54.5%) of the patients were male, and 263 (45.5%) – female. HRSV were detected in 154 (26.64%) samples: 55 (41.98%) were subtyped as HRSV-A and 71 (54.2%) as HRSV-B. The children were divided into 6 different age groups, shown in months (Figure 1).

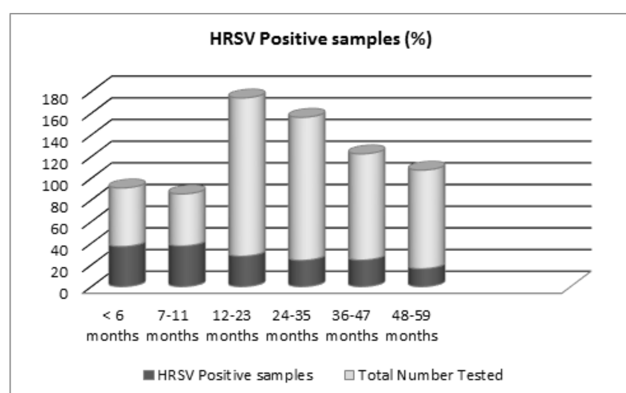

Figure 1. *Age distribution of HRSV positive samples (in percentage) among children in different age gorups during the winter epidemic seasons 2014/15 and 2015/16.*

The information on clinical diagnoses of the patients was provided by the physicians who send the specimens. Most (67.3%) patients presented with symptoms of ILI or ARI without complications, of these 21.85% were positive for HRSV infection. The contribution of HRSV in the development of complications to the lower respiratory tract (bronchiolitis, pneumonia) and to the central nervous system (CNS) (febrile seizures, oedema cerebri, aseptic meningitis, encephalopathy, and meningoencephalitis) was analyzed. HRSV was detected in 31 (50.8%) of cases of bronchiolitis and 30 (40%) of pneumonia (Table 2).

During the survey period human respiratory syncytial virus was found in 2 out of 6 children with asthma exacerbation and in 14 out of 41 patients with acute laryngitis/ laryngotracheitis.

Phylogenetic and Amino acid sequence analysis

Phylogenetic analysis of the G gene nucleotide sequences and deduced amino acid analysis were conducted on a total of 46 of HRSV positive samples. All analyzed Bulgarian RSV-A strains (n=21) belonged to the NA1 genotype, clustered into newly identified sub-genotype ON1 (Figure 2). The Bulgarian HRSV-A strains carried the characteristic duplication of 72 nucleotides, shifting the stop codon of the G gene in to position 322. The deduced amino acid sequences of the second hypervariable region of the G protein were analyzed in comparison with prototypic genotype strain ON67-1210A (JN257693). The predicted length of HRSV-A G protein was 321 amino acids due to duplication of 23 amino acids starting from position 285.

Only two putative N-glycosylation sites were identified on the second variable region of the G protein among the Bulgarian RSV-A strains – at positions 237 and 318. The substitution N318A was observed in three Bulgarian strains, while the N-glycosylation site at position 237 remain conserved. In the amino acid sequences of the analyzed HRSV-A G protein, 49 serine and threonine sites were found to be O-glycosylated.

Out of 25 analyzed HRSV subgroup B strains, 24 clustered into BA9 genotype and only 834/Bulgaria/2016 strain clustered into BA10 gengroup (Figure 3). The deduced amino acid sequences were compared to the prototypic for the BA gengroup BA3833/99B (AY333362). Duplication of 20 amino acids was observed in all Bulgarian HRSV-B strains. Amino acid mutations were seen at the following positions when compared to prototype strain: K218P, L223P, S247P, T254I, T270I, V271A, I281T and H287Y.

Two N-glycosylation sites were identified among the group B strains at positions 296 and 310 at the C-terminal end of the G protein gene, which are conserved among almost all

Table 2. *Distribution of detected HRSV cases according to clinical diagnosis of the patients.*

	Clinical diagnosis (No. of cases, %)			
	Bronchiolitis (n=61)	Pneumonia (n=75)	Neuroinfection (n=53)	ILI/ARI without complication (n=389)
HRSV-A	14	9	4	24
HRSV-B	15	14	3	35
Mixed infection HRSV-A+B	-	1	-	4
Unsubtyped	2	6	1	25
Total positive(n=154)	31 (50.8%)	30 (40%)	8 (15.1%)	85 (21.85%)

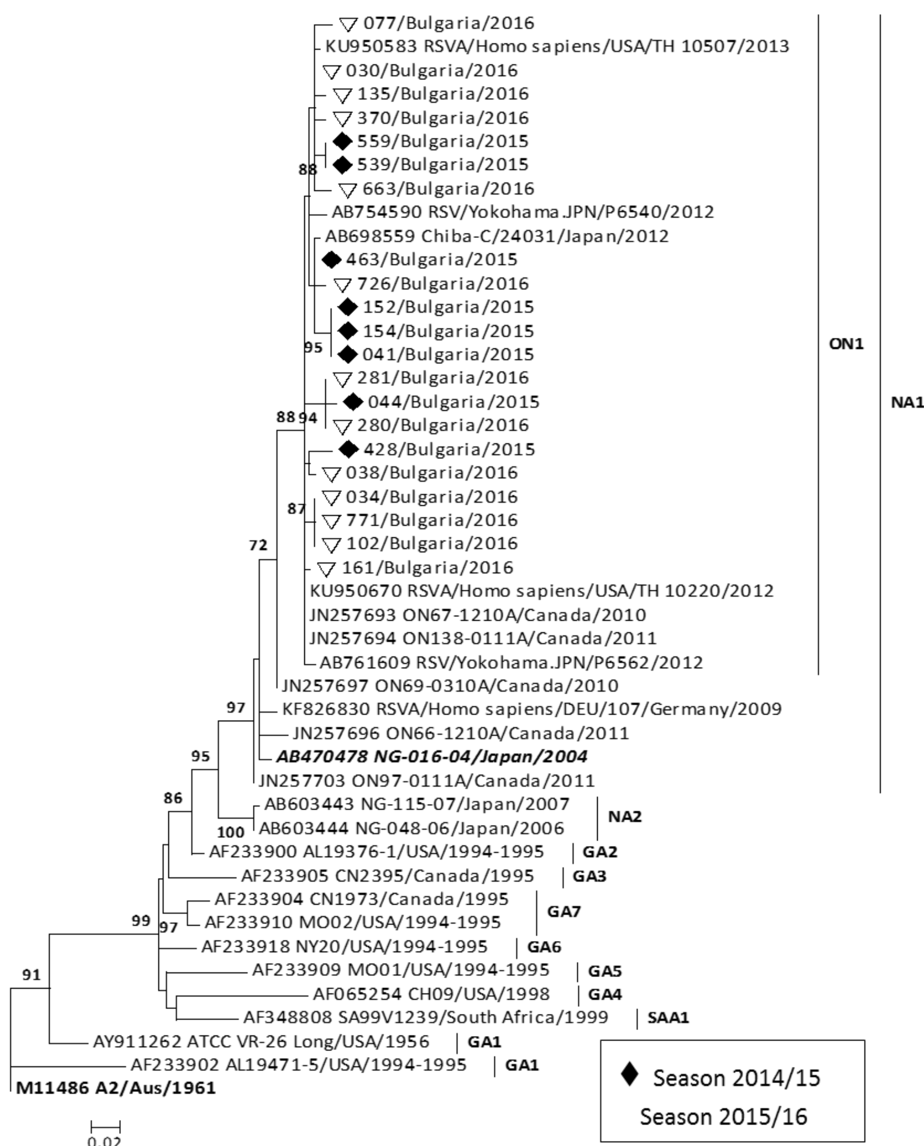

Figure 1. *Phylogenetic tree on the base of G gene of HRSV group A genotypes. The prototype strain A2 was used as the out group in the analysis. The tree was constructed using method based on the Tamura-Nei model with 1000 bootstrap replicates with MEGA, version 6 (Tamura, et al. 2013). The genotypes are indicated by the lines on the right side. Reference strain for the NA1 is shown in bold italic.*

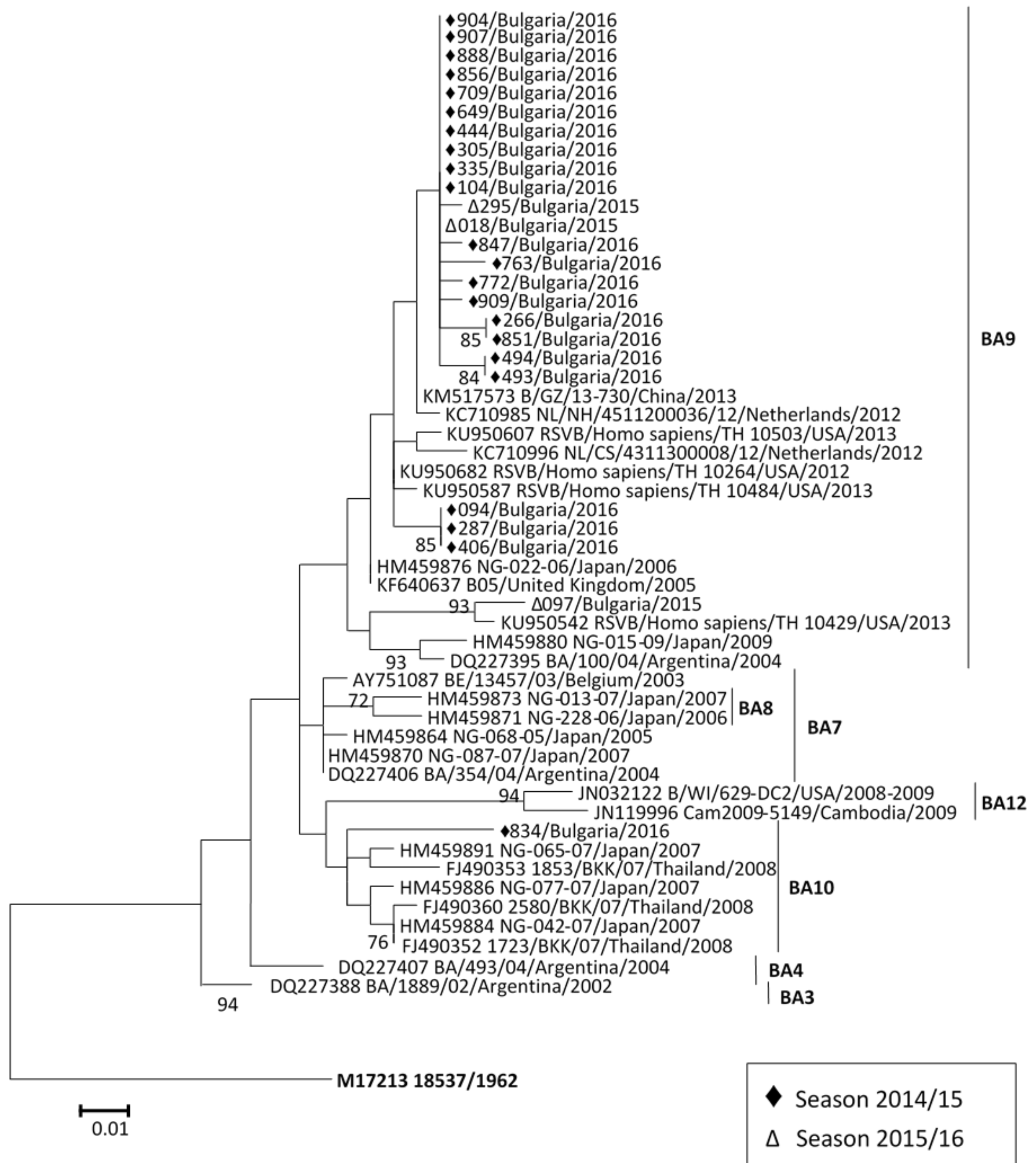

Figure 2. *Phylogenetic tree on the base of G gene of HRSV group B genotypes and subtypes. The prototype strain B 18537 was used as the out group in the analysis (bold). The tree was constructed using method based on the Hasegawa-Kishino-Yano model with 1000 bootstrap replicates with MEGA, version 6 (Tamura, et al. 2013). The genotypes are indicated by the lines on the right side. For visualization purposes some of the data originally included in the analysis were hidden. The complete phylogenetic tree is available upon request.*

the B strains. The N-glycosylation site at position 310 was missing in two of the Bulgarian strains.

There were 48 potential sites for O-linked sugars attachment at serine and threonine residues in the ectodomain of the analyzed HRSV-B G attachment protein.

Discussion

HRSV is one of the most common viral etiological agents of acute respiratory tract illness particularly in the early years of life leading to severe morbidity and hospitalization in very young children (C. Hall 2012). Various studies have reported the higher level of HRSV associated lower respiratory tract complications in infants less than six months of age, and in children under two years of age. Bronchiolitis and pneumonia were the most frequent clinical diagnoses in HRSV positive cases from our data.

A higher number of HRSV group B viruses - 71 (54.2%) were detected in comparison to group A strains (55 - 41.98%) in this study. Although there is a difference between the predominant HRSV subtypes in both investigated seasons, and during the season 2014/15 a higher incidence of HRSV-A positive cases was observed. On the basis of our limited data, we cannot make a comparison of the distribution pattern of HRSV-A and B subtypes at present but ongoing surveillance data will be able to better elucidate the prevalent pattern.

The present study is the first one in Bulgaria which gives detailed data on molecular characteristics of HRSV associated ARI and ILI in Bulgarian children. We analyzed G protein sequences of HRSV-A and B strains detected in clinical samples of children less than 5 years of age during two consecutive epidemic seasons 2014/15 and 2015/16. The HRSV-A G protein was found to be 321 amino acids in length, unlike the reference strain A2 which has a length of 298 aa. This is due to duplication of 72 nucleotides, corresponding to duplication of 23 amino acids. The observed duplication is characteristic for the ON1 genotype. The distinctive substitutions E232G, T253K and P314L were found in all amino acid sequences analyzed. Our data suggests that HRSV-A strains recently circulating in Bulgaria clustered with ON1 genotype which originated in Canada and spread globally (Auksornikitti, et al. 2014, Eshaghi, et al. 2012).

In all analyzed Bulgarian group B strains was observed a signature for the BA genotype 60-nucleotide duplication in the HVR2 of the G protein. The BA genotype was first isolated in 1999 in Buenos Aires, Argentina (Galiano, et al. 2005) and subsequently was diverged into genetic groups BA1-BA12 (Auksornikitti, et al. 2014, Gimferrer, et al. 2015, Khor, et al. 2013, Martinelli, et al. 2014, Tran, et al. 2013). The BA10-speciffic substitutions were observed in only one Bulgarian RSV-B strain. All other HRSV subgroup B strains clustered into BA9 genotype.

N- and O-linked glycosylation sites can influence the expression of certain epitopes by either masking or contributing to recognition by specific antibodies and thus to help viruses to escape from the host immune response (Hansen, et al. 1998). The analyses of the amino acid sequences at glycosylation sites will help to understand mechanisms that give selective advantage for viruses.

The present study highlights the significance of HRSV as a dominant viral etiologic agent of pediatric ARI and further work to identify the circulation pattern of HRSV strains in Bulgaria, is needed. Long-term molecular surveys are important for early detection of prevalent strains and newly emerging genotypes.

Acknowledgement

This study was funded by the Bulgarian Ministry of Health and National Influenza Pandemic Preparedness Plan.

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