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Characterization of post-pandemic influenza virus circulation in southern region of Romania during 2010–2014

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1. Introduction

In April 2009 the CDC (Center for Disease Control and Prevention) identified in Mexico two cases infected with a swine-origin influenza virus (H1N1) with a never seen before genetic composition that rapidly spread around the globe and cause the first influenza pandemic of the 21st century (CDC Novel H1N1 Flu|The 2009 H1N1 Pandemic: Summary Highlights, n.d.; Girard et al., 2010). The novel pandemic virus was named A/(H1N1)pdm09, and it was the product of a triple reassortment between swine circulating North American H3N2 triple reassortant viruses, H1N1 classic swine virus and a H1N1 with avian-like characteristics that circulated in Europe and Asia (Smith et al., 2009).

In Romania, the first cases of A(H1N1)pdm09 were detected in June 2009 and from that moment on it was in complete dominance for the rest of 2009 and lasted until March 2010. The pandemic peak was registered much earlier (week 48/2009) than in an influenza epidemic season, and the incidence level was also much higher (32,6 per 100.000 population). Also, unlike a seasonal influenza virus, the A/(H1N1) pdm09 subtype mainly affecting the 30–34 y.o. age group and caused 122 deaths (Communicable diseases surveillance report for 2009 [Internet], n.d.).

The World Health Organization (WHO) declared in August 2010 the end of the most recent pandemic and the post-pandemic phase started. During the post-pandemic phase the A(H1N1)pdm09 virus characteristics resembled more and more to a seasonal virus: the intensity decreased to seasonal levels, spread patterns were different, out-of-season outbreaks were no longer detected and a mixed influenza circulation was reported in many countries (WHO recommendations for the post-pandemic period, n.d.). Severe cases occurred at lower levels than during the pandemic, while the risk groups remained the same (young children, pregnant women and those with respiratory or chronic health conditions, including asthma, diabetes or obesity).

In Romania the post-pandemic seasons were mainly characterized by a mixed influenza picture of A(H1N1)pdm09 co-circulation with either A/H3N2 or influenza B, with the exception of season 2011–2012 when A/H3N2 clearly dominated, but was largely representative for the rest of Romania and Central and East European countries.

All four post-pandemic influenza seasons started relatively early, between week 44–47, if compared with pre-pandemic seasons, they peaked between weeks 8–10 and ended between weeks 13–17. With the exception of the first season when the most affected age group was 30–64 y.o., in the rest of the seasons the dominant age group affected was 15–49 y.o. The regional incidence rates (%000 inhabitants) ranged between: 0.3–1.151 for A/(H1N1)pdm09, 0.364–1.092 for A/H3N2 and 0.157–1.043 for influenza B. The highest mortality rate for ILI cases was recorded in season 2010–2011 at 3.47% while for SARI was 12.5% in season 2013–2014.

The aim of this paper is to provide a comprehensive picture of influenza virus genetic and antigenic characteristics during the first four post-pandemic seasons in South Romania, and highlights the fact that influenza is an important cause of morbidity and mortality each year that should be mitigated by vaccination.

2. Materials and methods

2.1. Study population and specimen collection

The paper analyzes the first four post-pandemic seasons, between 2010 and 2014, in the southern region of Romania, inhabited by just over 10 million people (roughly half of Romania population), organized into 18 counties and the capital city of Bucharest. This region is under the public health management of the Regional Center of Public Health Bucharest, within the National Institute of Public Health, together with the rest of the country. In Romania, influenza surveillance is coordinated by the National Institute of Public Health (NIPH) in

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cooperation with the National and Regional Centers, and the local public health authorities. Nation-wide laboratory surveillance of Influenza is performed at the National Influenza Centre (NIC), based in the National Research Institute "Cantacuzino". The surveillance targeted influenza like illnesses (ILI), acute respiratory illnesses (ARI), severe acute respiratory illnesses (SARI) and viral detections, through routine and sentinel systems. The ILI and ARI sentinel network - built under the WHO recommendations of representativeness: minimum 2% of the population and 1% of the family medicine practitioners, 30% of them from rural healthcare units - enrolled 98 general practitioners (GPs) of the primary health care system distributed across 15 counties from South Romania. The SARI sentinel network enrolled up to 10 hospitals from Bucharest and three other counties of South Romania (Brasov, Constanta, Dolj). The weekly number of ILI, ARI and SARI cases are reported to the Ministry of Health and further reported to ECDC (European Centre for Disease Prevention and Control). Respiratory specimens were systematically collected according to national influenza surveillance methodology and ECDC case definitions for ILI and SARI (2012/506/EU, 2012). Nasopharyngeal swabs were stored at 4 °C for up to 72 h before shipment and RNA extraction was performed upon receiving the sample or were stored at −80 °C.

2.2. Statistics

Data analysis was performed with Epiinfo and MS Excel applications, targeting the following indicators: the notified ILI and SARI cases and influenza virus molecular detections by type and subtype.

2.3. Molecular detection of influenza viruses

Briefly, viral RNA is purified from clinical specimens or from isolated samples using commercial kits, such as NucleoSpin viral RNA virus Kit (Macherey-Nagel Gmbh, Germany). Real time RT-PCR was performed with Superscript III Platinum One-step qRT-PCR System (Invitrogen, Life Technologies, USA) with specific primers (provided by National Institute for Public Health and the Environment, Bilthoven and CDC Atlanta) targeting the matrix genes of influenza A and B viruses. For influenza A, a second RT-PCR was performed in order to differentiate between subtypes A(H1N1)pdm09 and A/H3N2. The rRT-PCR protocol for detection of influenza A, B and subtype H3 constitutes of reverse transcription at 55 °C for 30 min, Taq inhibitor inactivation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/amplification at 60 °C for 30 s. The H1 pdm09 detection protocol presents two modifications of the previous protocol: reverse transcription at 55 °C for 30 min and annealing/amplification at 55 °C for 30 s. A C_t value < 37 was regarded as positive. Amplification was performed on a Stratagene Mx3005P Multiplex Quantitative PCR System (Agilent Technologies, Santa Clara, CA, USA). A second rRT-PCR was also performed for influenza B lineage determination between Yamagata and Victoria, either for all or a fraction of positive samples detected at specific influenza epidemic time points (i.e. start, plateau and decline phase) (Cherciu et al., 2014).

2.4. Influenza antigenic characterization

Phenotypic analysis consists of hemagglutination inhibition assay (HI) as described elsewhere (Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza, 2011) was performed on viral supernatant of Madin-Darby canine kidney (MDCK) cells or allantoic fluid of hen's embryonated eggs to evaluate the recognition efficacy of the circulating influenza viruses by the current influenza vaccine-induced antibodies on guinea pig erythrocytes or turkey red blood cells (RBC). For subtype H3N2, the hemagglutination inhibition assay was performed in the presence of 20 nM oseltamivir in order to circumvent possible agglutination of RBCs mediated by the virus neuraminidase (Lin et al., 2012). Reference post-infection ferret antisera provided by the WHO CC for Reference and Research on Influenza (MRC NIMR, London, UK and CDC Atlanta).

2.5. Genetic characterization

Full frame sequencing of hemagglutinin and neuraminidase encoding genes was performed with specific primers (sequences provided by PHE, London, UK) that amplifies two or three overlapping gene segments directly from original sample for specimens identified by rRT-PCR (C_t < 30) were possible, or from virus isolate. Revers transcription was performed with UNI12 influenza universal primer and Invitrogen Platinum Superscript III RT kit (Carlsbad, CA, USA). Invitrogen Platinum Pfx DNA Polymerase kit was used for PCR amplification. Gel purification was performed with Macherey-Nagel NucleoSpin Gel and PCR Clean-up (Düren, Germany). Sequencing was performed with BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems Foster City, California) used in accordance with the manufacturer's instructions. DNA cleanup was performed with Qiagen DyeEx 2.0 Spin Kit (QIAGEN, Hilden, Germany) which uses spin columns to remove unincorporated dye terminators from sequencing reactions. Finally capillary electrophoresis was performed on a four-capillary ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems Foster City, California).

The Maximum Likelihood phylogenetic trees were constructed using RAxML (Stamatakis, 2014) version 8.0.0 with the GTR-GAMMA nucleotide substitution model. The tree branch reliability was evaluated with 1000 bootstrap replicates. Phylogenetic trees were drawn using FigTree (Rambaut, 2012) and edited further with Inkscape. All the influenza virus nucleotide sequences used to construct the phylogenetic trees were retrieved from GISAID genetic database.

2.6. Antiviral susceptibility surveillance

The NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems, Foster City, CA, USA) was used to measure the resistance level (IC_{50}) of influenza virus isolates to neuraminidase inhibitor antivirals (i.e. Oseltamivir) in accordance with the manufacturer's instructions. Briefly, serial virus dilutions from cell culture supernatant were incubated for a short time with neuraminidase inhibitor, and then incubated for 10–30 min with NA-Star 1,2 – dioxetane chemiluminescent substrate. Chemiluminescence was read from assay plates with TriStar LB 941 multimode reader (Berthold Technologies, Bad Wildbad, Germany). IC_{50} values for viral isolates were determined by performing a dose-response analysis using non-linear regression with GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA). Reference strains A/Mississippi/3/01 (sensitive, H274) and A/Mississippi/3/01 (resistant, 274Y) were used to assess the level of A(H1N1)pdm09 isolates susceptibility to oseltamivir, while reference strains A/Fukui/20/04 (sensitive, E119) and A/Fukui/45/04 (resistant, 119 V) were used for H3N2 isolates. Isolates with IC_{50} values above the base line were further characterized by sequencing the full frame neuraminidase gene, which can identify specific mutation(s) associated with Oseltamivir and Zanamivir resistance and can be used to investigate novel resistance mutations (Necula et al., 2011).

3. Results

During the first four post-pandemic seasons the notified ILI cases ranged between 500 and 4000, while SARI cases were between 87 and 240 (Table 1). rRT-PCR was performed for 14–55% (300–600) of the ILI cases and 92–97% (80–220) of SARI cases. The overall influenza virus positive detections ranged between 28 and 55% for ILI cases and between 31 and 48% for SARI cases. An overview of the four post-pandemic seasons in South Romania shows, in general, a mixed influenza virus circulation: Co-circulation of A(H1N1)pdm09 subtype and B type

Table 1
Influenza notified cases, molecular detections and detection rates in South Romania, 2010–2014.

	ILI				SARI			
	2010–11	2011–12	2012–13	2013–14	2010–11	2011–12	2012–13	2013–14
No of cases detected	4190	915	979	541	240	141	208	87
Tested (%)	14	34	47	55	97	92	94	95
Influenza positive (%)	30.2	28	48	55	48	31	47	41
A(H1N1pdm09) (%)	40.7	0	39	31.7	48.4	0	63.8	54.8
A(H3N2) (%)	0	95.6	5.1	68.3	0.8	100	1.7	45.2
Type B (%)	59.3	4.3	55.9	7.4	50.8	0	34.5	0

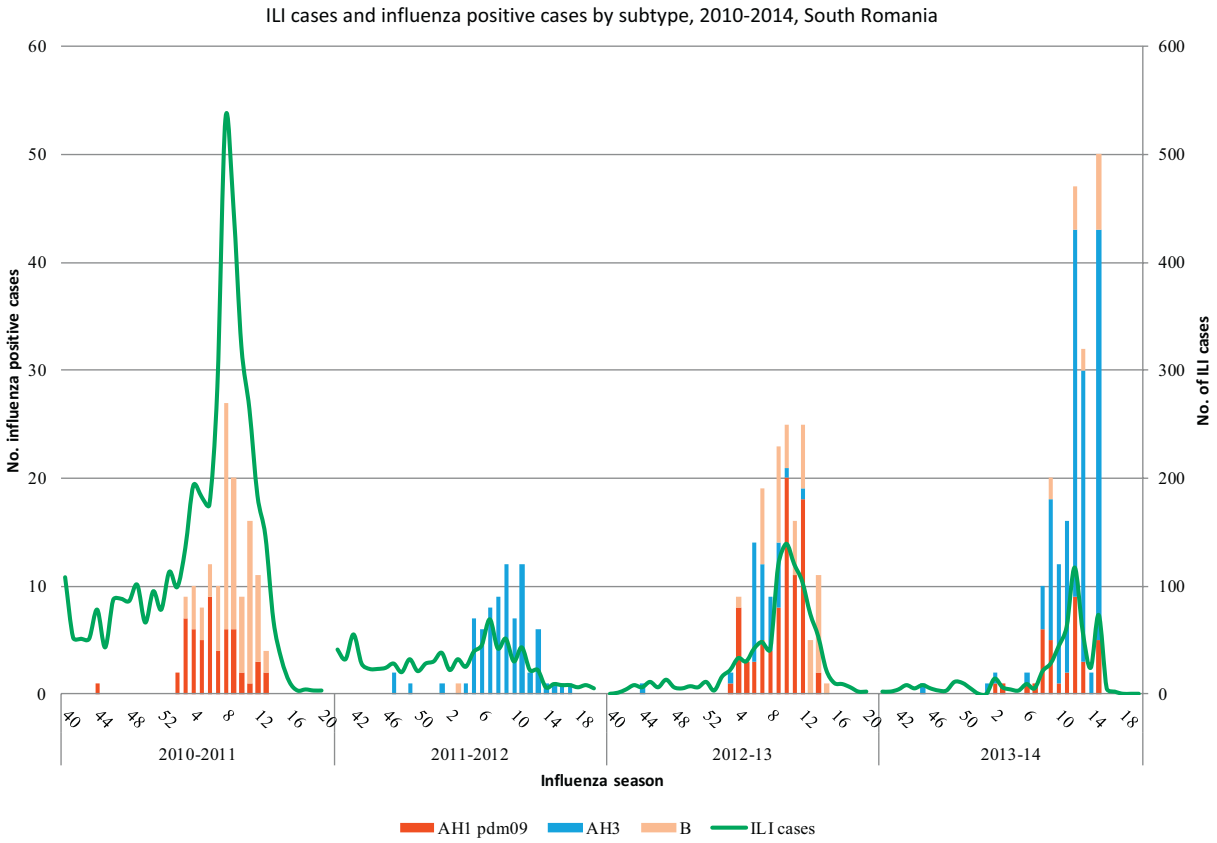

Fig. 1. Weekly ILI cases, South Romania, 2010–2014.

in seasons 2010–2011 and 2012–2013, co-circulation of A(H1N1) pdm09 and A/H3N2 subtypes in season 2011–12, and a strong dominance of A/H3N2 in season 2011–2012 (Fig. 1). The median age of severe cases associated with A(H1N1)pdm09 infection between 2010 and 2014 (101), was of 39.5 years and the sex ratio (M/F) was 1.2:1. A total of 67 strains were isolated from South Romania, 43 from ILI and 24 from SARI cases. All the isolates manifested normal susceptibility to antivirals (Oseltamivir and Zanamivir).

The 2010–2011 season started in week 44/2010, peaked in week 8/2011 and ended in week 13/2011. The most affected age group was the 30–64 y.o. (38.73%). The overall influenza fatality rate was 3.47%, while the regional incidence rates (%000 inhabitants) were: 1.043 for influenza B virus and 0.649 for the A/(H1N1)pdm09 subtype. In the first post-pandemic season a marginal dominance of influenza B (59%) versus subtype A(H1N1)pdm09 (41%) was registered, while the subtype A/H3N2 was uncommon, with a single detection in South Romania (Constanta county) from a SARI case. All influenza B viruses characterized by HI assay belonged to the B/Victoria/16/88 lineage (B/Brisbane/60/2008 –like). The circulating viruses had similar antigenicity to those of previous years, and were closely related to the three strains contained in the seasonal influenza vaccine. All A/(H1N1) pdm09 strains sequenced carried the substitutions P83S and S203 T in the HA gene, while D97N and S185 T were present in 80%, respectively 70% of sequences. A single strain from a SARI case contained the mutation D222N. Mutations S185 T, S143G are located within or adjacent to HA antigenic sites, while D222N is located in the receptor binding site (RBS), and were previously reported in viruses from the 2010–2011 season in Europe and other parts of the world. (Fig. 3). For SARI 44.35% of cases were influenza positive (106/239): 50.9% A/(H1N1) pdm09, 48.1% influenza B and only 0.9% A/H3N2. Out of all SARI cases tested 3.3% were non-influenza detections. The most affected group was 30–64 y.o. (38%), followed by 15–29 y.o. (23.6%), and fatality rate of 4.72%. The regional incidence rates were 2.6 for A/(H1N1)pdm09 and 2.5 for influenza B.

The 2011–2012 season started in started in week 47/2011, peaked in week 9/2012 and ended in week 17/2012. The most affected age group was 15–49 y.o. (59%), and the regional incidence rate 0.689 (%000 inhabitants) for the A/H3N2 virus. In the second post-pandemic season (2011 – 2012), influenza transmission was primarily associated with influenza A/H3N2 throughout most of the season, representing

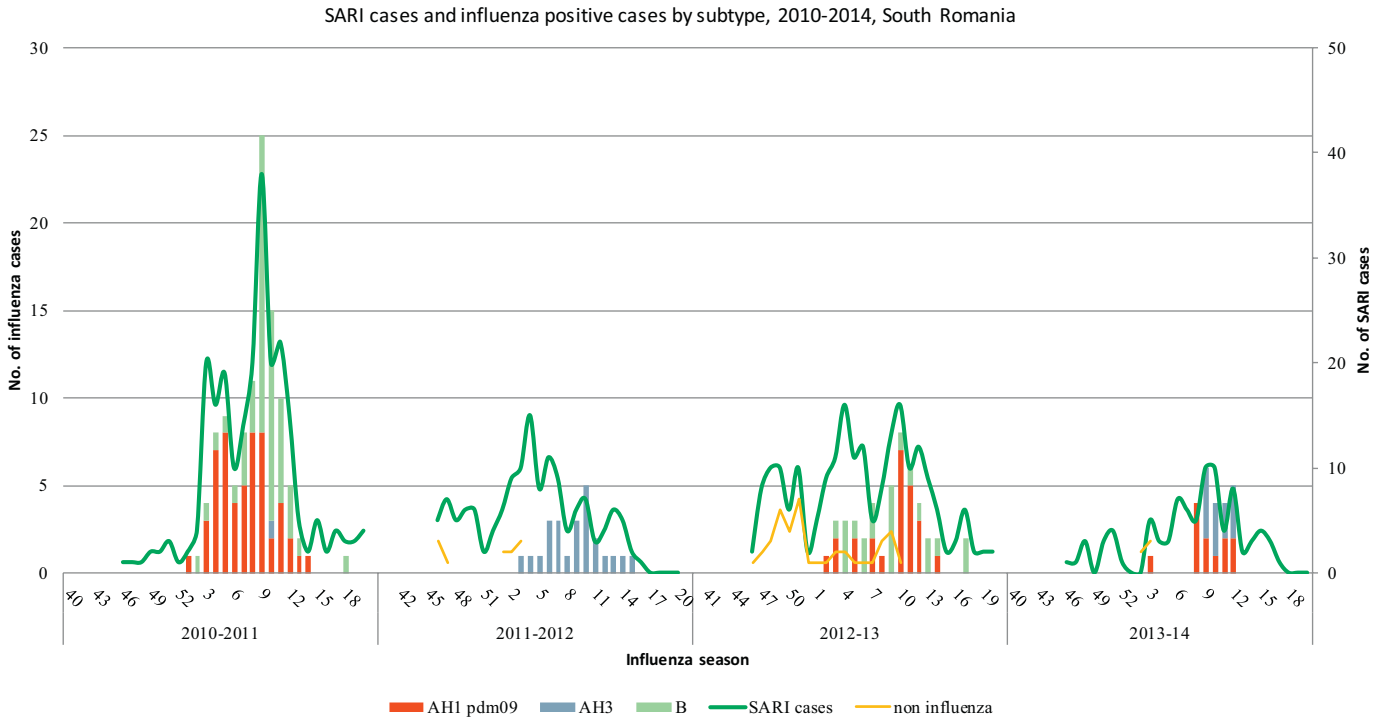

Fig. 2. Weekly SARI cases, South Romania, 2010–2014.

98.8% of all detections in South Romania, with a single influenza type B detected. All A/H3N2 viruses isolated showed a reduction of titer in the HI assay of ≥4-fold with serum raised against the vaccine virus A/Perth/16/2009. Genetic characterization (HA gene) of 20 A(H3N2) strains revealed that all fell in Victoria/208 genetic clade Fig. 4. All HA sequences had the following substitutions: K62E, K144N (potential glycosylation site) and T212A – shared with Perth/16 genetic clade. Phylogenetic analysis of HA genes revealed that 14 strains belong to genetic group 6 (prototype strain A/Iowa/19/2010), 5 strains fell in genetic group 3C (A/Hong Kong/3969/2011), and a single strain fell in genetic group 3A (A/Stockholm/18/2011). All four A/H3N2 NA gene sequences had two common substitutions: S367 N and K369 T that are not associated with antiviral resistance. For SARI 17% samples were influenza positive (24/141): all A/H3N2, while no A/(H1N1)pdm09 and influenza B cases were detected. Non-influenza virus detections were 14.1% of SARI cases. The age groups affected were 2–4 y.o. (25%), followed by 0–1 y.o. (21%), and fatality rate of 0%. Regional incidence rates were 0.935 for A/H3N2 and 0.246 for influenza B.

The 2012–2013 season started in week 44/2012, peaked in week 10/2013 and ended in week 15/2013. The most affected age group 15–49 y.o. (49%). The regional incidence rates 1.151 for the A/(H1N1)pdm09 subtype, 0.482 for influenza B and 0.364 for A/H3N2. In the third post pandemic season (2012 – 2013), 56% of positive clinical specimens were influenza B and 44% influenza A. Of the influenza A viruses, 88.4% were A/(H1N1)pdm09, including a co-infection with A(H1N1)pdm09 and Influenza B in a SARI patient, and 11.6% A/H3N2. Although A/H3N2 subtype was relatively uncommon in this season, and presented a considerable degree of difficulty to isolate on non-MDCK-SIAT cells, the first isolate A/Arges/126697/12, week 43/2012, was from South Romania. The HI assay showed considerable heterogeneity to the antiserum raised against A/Victoria/361/2012 (cell culture or egg-propagated), or antiserum raised against the recommended vaccine virus strain (A/Texas/50/2012) – a virus antigenically similar to the cell-propagated prototype virus A/Victoria/361/2011. The antigenic characterization of A/(H1N1)pdm09 isolates showed good match with the vaccine strain recommended by WHO for inclusion in the 2012/13 northern hemisphere seasonal influenza vaccine composition.

The dominating type B virus belonged to B/Yamagata/16/88 lineage with a good reactivity with B/Wisconsin/1/2010 vaccine prototype but also the isolates reacted well (titers within 4-fold of the homologous titers) with B/Massachusetts/2/12 – the vaccine strain also recommended for inclusion in vaccine composition of 2013–2014 season. HA gene sequencing of 8 influenza B isolates (Yamagata-lineage) revealed that all fell in clade 2, (B/Estonia/55669/2011), together with vaccine strain B/Massachusetts/2/12. A single isolate (B/Iasi/131732/2013) belonged to Victoria-lineage (clade 1A), marked mainly by the absence of substitution L58P (Fig. 5). All four A/H3N2 strains sequenced fell in group 3C (A/Victoria/361/2011) with the following common substitutions: Q33R, N145S and N278K. All 11 A(H1N1)pdm09 strains sequenced fell in group 6 (A/St Petersburg/27/2011). None of NA gene sequenced from 10 A/(H1N1)pdm09, 2 A/H3N2 and 2 type B strains revealed the presence of substitutions associated with reduced antiviral susceptibility. For SARI cases 22.1% were influenza positive (46/208): 52.2% A/(H1N1)pdm09, 47.8% influenza B, and 21.1% non-influenza detections. The most affected age groups were 15–49 y.o. (30.43%), followed by 50–64 y.o. and 5–14 y.o. (26.09% each), while fatality rate was 6.52%. The regional incidence was 1.377 for A/(H1N1)pdm09 and 0.886 for influenza B.

The 2013–2014 season started in week 45/2013, peaked in week 9/2014 and ended in week 15/2014. The the most affected age group was 15–49 y.o. (45%). The overall influenza fatality rate was 1.91%, and the regional incidence rates were: 1.092 for A/H3N2 virus, 0.3 for A/(H1N1)pdm09 and 0.157 for influenza B. The dominant virus type in the season 2013–2014 was influenza A (96.2%), while type B circulated sparingly (3.8%). Subtype A/H3N2 represented 63.2%, and A/(H1N1)pdm09 36.8% of influenza A. Antigenic characterization of A/(H1N1)pdm09 and A/H3N2 isolates closely matched with the corresponding strains recommended by WHO for inclusion in the 2013/14 northern hemisphere seasonal influenza vaccine: A/California/7/09 and A/Texas/50/2012. All 13 A/H3N2 strains from South Romania fell in subclade 3C (A/Texas/50/2012). Of these, four fell in sub-group 3C.2 (A/Ireland/M28390/2013) while 9 fell in sub-group 3C.3 (A/Samara/73/2013). Four strains from sub-group 3C.3 additionally had the

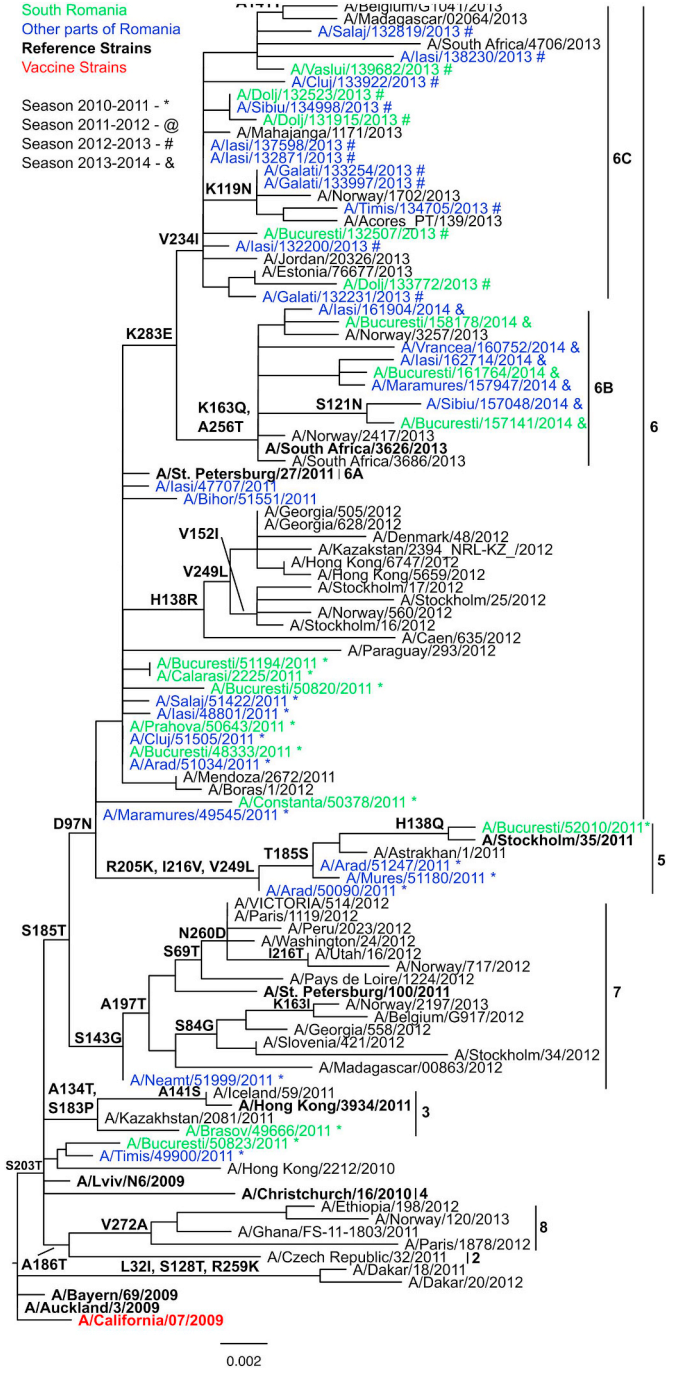

Fig. 3. Phylogenetic tree of influenza A(H1N1)pdm09 HA genes from the South Romania and the rest of the country in relation to sequences from rest of the Europe. GISAID accession numbers: A/Timis/49900/2011 EPI317501; A/Maramures/49545/2011 EPI317502; A/Bucuresti/48333/2011 EPI317503; A/Cluj/51505/2011 EPI317504; A/Salaj/51422/2011 EPI317505; A/Mures/51180/2011 EPI317506; A/Bucuresti/51194/2011 EPI317507; A/Calarasi/2225/2011 EPI317508; A/Arad/50090/2011 EPI317509; A/Prahova/50643/2011 EPI317510; A/Iasi/48801/2011 EPI317511; A/Neamt/51999/2011 EPI317512; A/Arad/51247/2011 EPI317518; A/Constanta/50378/2011 EPI317525; A/Bucuresti/52010/2011 EPI317528; A/Bihor/51551/2011 EPI317529; A/Brasov/49666/2011 EPI317530; A/Arad/51034/2011 EPI317531; A/Bucuresti/50820/2011 EPI317532; A/Bucuresti/50823/2011 EPI317533; A/Dolj/132523/2013 EPI425970; A/Iasi/132200/2013 EPI439283; A/Dolj/131915/2013 EPI439285; A/Galati/132231/2013 EPI439287; A/Bucuresti/132507/2013 EPI439289; A/Salaj/132819/2013 EPI439291; A/Galati/133254/2013 EPI439295; A/Galati/133997/2013 EPI439302; A/Cluj/133922/2013 EPI439304; A/Dolj/133772/2013 EPI439306; A/Iasi/132871/2013 EPI439360; A/Iasi/138230/2013 EPI464961; A/Bucuresti/157141/2014 EPI509348; A/Sibiu/157048/2013 EPI509349; A/Maramures/157947/2014 EPI509351; A/Bucuresti/158178/2014 EPI509352; A/Iasi/161904/2014 EPI516548; A/Bucuresti/161764/2014 EPI516549; A/Vrancea/160752/2014 EPI516550; A/Iasi/162714/2014 EPI516551; A/Iasi/47707/2011 EPI309659; A/Vaslui/139682/2013 EPI460725; A/Timis/134705/2013 EPI460723; A/Sibiu/134998/2013 EPI460697; A/Iasi/137598/2013 EPI460664.

substitution N122D, while six viruses share the substitution L157S. All eight A/(H1N1)pdm09 strains fell in sub-clade 6B, and shared the substitutions K163Q, A256T and K283E (A/Norway/2417/2013). The four influenza type B strains sequenced were of B/Yamagata/16/88 lineage, and fell in genetic clade 3 represented by a previous vaccine virus (B/Wisconsin/1/2010 and reference viruses B/Stockholm/12/2011) while the included influenza B vaccine strain for the northern hemisphere is B/Massachusetts/02/2012 (clade 2). The genetic and antigenic characterization of A(H1N1)pdm09 and H3N2 viruses revealed close match with the viruses recommended by WHO for inclusion in the 2013/14 northern hemisphere seasonal influenza vaccine. The circulating type B matched poorly with the included vaccine virus (B/Massachusetts/02/2012), although detection level was low in Romania in this season. Sequencing of the neuraminidase gene from eight strains (5 A/H3N2, 2 A/(H1N1)pdm09 and 1 type B) did not reveal the presence of substitutions associated with reduced antiviral susceptibility.

During the four post-pandemic seasons we have registered 641 SARI cases, of which 101 testing positive for A/(H1N1)pdm09 subtype, their median age was 39.5 years, mostly affecting the age group 15–49 y. o. Most of A/(H1N1)pdm09 positive cases (80%) presented the following comorbidities: obesity (33%), heart and blood vessels chronic diseases (31%), lung chronic diseases (18.5%), diabetes (16%) and 1 case of pregnancy (0.012%). For SARI 27.5% were influenza positive cases (24/87) evenly split between A/(H1N1)pdm09 and A/H3N2 and 10.3% cases tested positive for non-influenza viruses. The most affected age groups were 15–49 y.o. (29%), followed by 65+ y.o. (21%), and the highest fatality rate of the four seasons at 12.5%. The regional incidence rates were 0.590 for both A/(H1N1)pdm09 and A/H3N2.

4. Discussion

During the pre-pandemic seasons, in the southern region of Romania the routine system registered about 500 ILI cases, peaking regularly in week 6–10, but during the 2009–2010 pandemic this volume increased > 8 times (4375 ILI cases), peaking much earlier (week 50/2009). It took 4 post-pandemic seasons until the number of cases turned back to the pre-pandemic level, while the peaking turned back from the very first post-pandemic season to week 8–12. The post-pandemic descending dynamic was more obvious in ILI than in SARI cases: 4 to 8 times seasonal decreasing in comparison to the first post-pandemic season for ILI cases, and up to 3 times in comparison to the first post-pandemic season for SARI cases. The profile of detected influenza virus shifted from the complete dominance of A/(H1N1)pdm09 virus to co-circulation with either influenza B or A/H3N2. Young adults were the most affected by severe infections with the A/(H1N1)pdm09 subtype, half of them presenting comorbidities: obesity or heart and blood chronic diseases.

During the 2010–2011 season, the subtype A(H1N1)pdm09 was the most frequently detected virus in Europe but it co-circulated with influenza type B. Romania and several European countries: Hungary, Slovenia and the UK registered higher rates of ILI and/or ARI during the 2010–2011 season than during the pandemic (2009–2010) (European Centre for Disease Prevention and Control, 2011). The A/(H1N1)pdm09 was the dominant virus until week 6 of 2011, when it was superseded by influenza type B, and continued to have a diminished presence, while influenza B continued to rise (Fig. 2). There was a delay

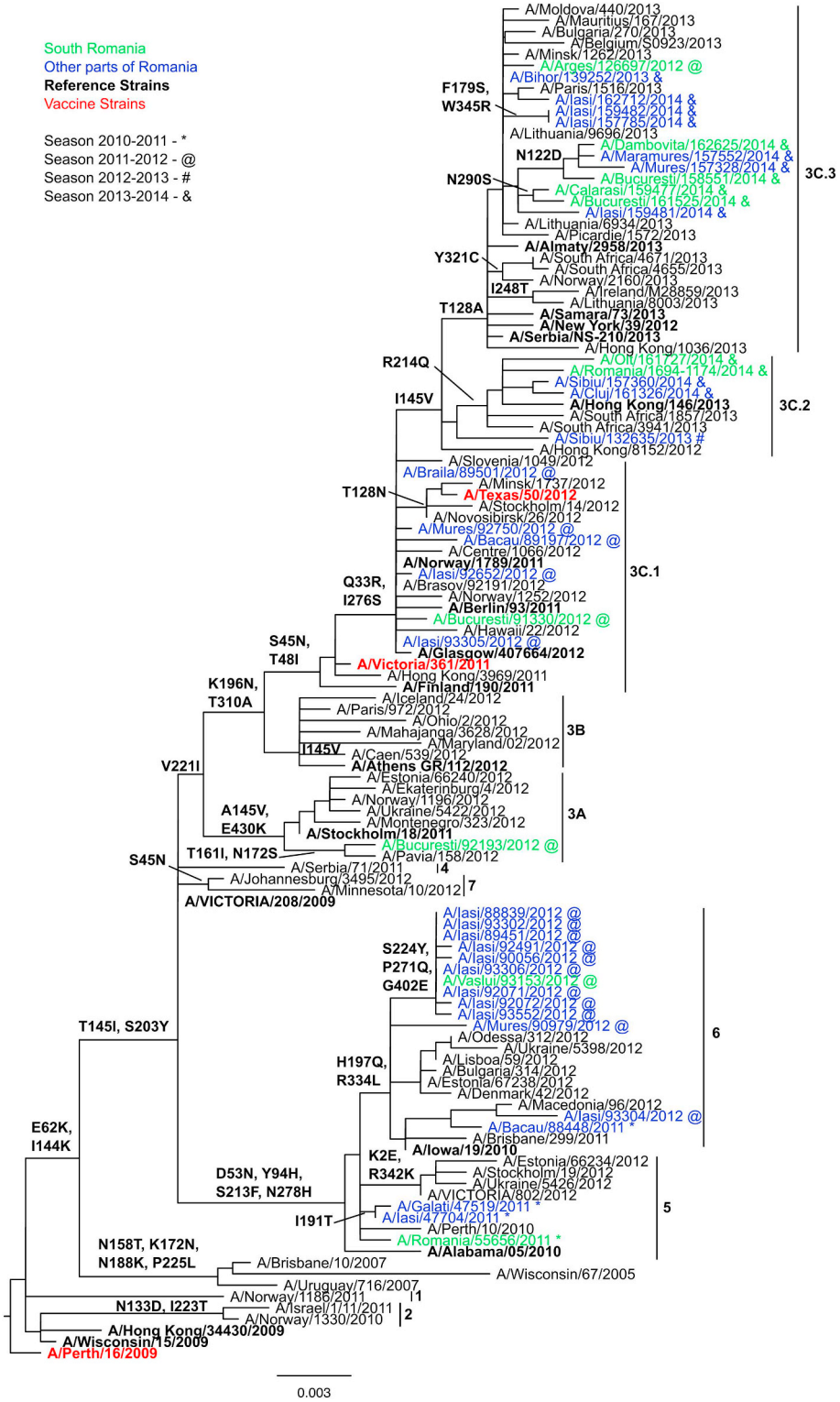

Fig. 4. Phylogenetic tree of influenza H3N2 HA genes from the South Romania and the rest of the country in relation to sequences from rest of the Europe. GISAID accession numbers: A/Galati/47519/2011 EPI309727; A/Iasi/47704/2011 EPI309733; A/Romania/55656/2011 EPI319302; A/Braila/89501/2012 EPI358915; A/Bacau/88448/2011 EPI370334; A/Iasi/90056/2012 EPI370456; A/Iasi/89451/2012 EPI370457; A/Mures/90979/2012 EPI370458; A/Bucuresti/91330/2012 EPI370459; A/Iasi/92071/2012 EPI370460; A/Brasov/92191/2012 EPI370461; A/Bucuresti/92193/2012 EPI370462; A/Iasi/92491/2012 EPI370463; A/Iasi/93304/2012 EPI370464; A/Iasi/93305/2012 EPI370465; A/Iasi/93552/2012 EPI370466; A/Iasi/92652/2012 EPI370467; A/Mures/92750/2012 EPI370468; A/Vaslui/93153/2012 EPI370469; A/Iasi/92072/2012 EPI370482; A/Bacau/89197/2012 EPI370483; A/Iasi/93302/2012 EPI370484; A/Iasi/88839/2012 EPI370486; A/Iasi/93306/2012 EPI370488; A/Arges/126697/2012 EPI416328; A/Sibiu/132635/2013 EPI439297; A/Bihor/139252/2013 EPI464967; A/Mures/157328/2014 EPI500259; A/Sibiu/157360/2014 EPI500261; A/Maramures/157552/2014 EPI500263; A/Iasi/157785/2014 EPI503288; A/Romania/1694-1174/2014 EPI509353; A/Bucuresti/158551/2014 EPI509355; A/Calarasi/159477/2014 EPI509356; A/Iasi/159482/2014 EPI509357; A/Iasi/159481/2014 EPI509358; A/Dambovita/162625/2014 EPI516543; A/Bucuresti/161525/2014 EPI516544; A/Cluj/161326/2014 EPI516545; A/Iasi/162712/2014 EPI516546; A/Olt/161727/2014 EPI516547.

in the general peak of detections in Romania (week 10/2011) and to a lesser extent South Romania (8/2011), compared to the majority of European countries (week 6/2011).

The 2011–2012 influenza season started unusually late, with an unclear pattern of geographical spread, and with the exception of few Western European countries it had a mild intensity. In Romania, and other European countries: Czech Republic, Denmark, Lithuania, Slovakia and the United Kingdom, the intensity was noticeably lower than in the 2010/11 season (Fig. 2) (European Centre for Disease Prevention and Control, 2012). As in the rest of Europe, the A/H3N2 was the dominant virus in Romania during the 2011/12 influenza season. The two influenza type B lineages were detected in relative similar level across Europe, while in Romania only B/Victoria/2/87 was detected (European Centre for Disease Prevention and Control, 2012). Antigenic and genetic characterizations of the A/H3N2 subtype indicated a high degree of virus diversity that translated in less than optimal vaccine match. Influenza vaccine effectiveness (IVE) studies of the time indicated an effectiveness of only 25% among all age groups

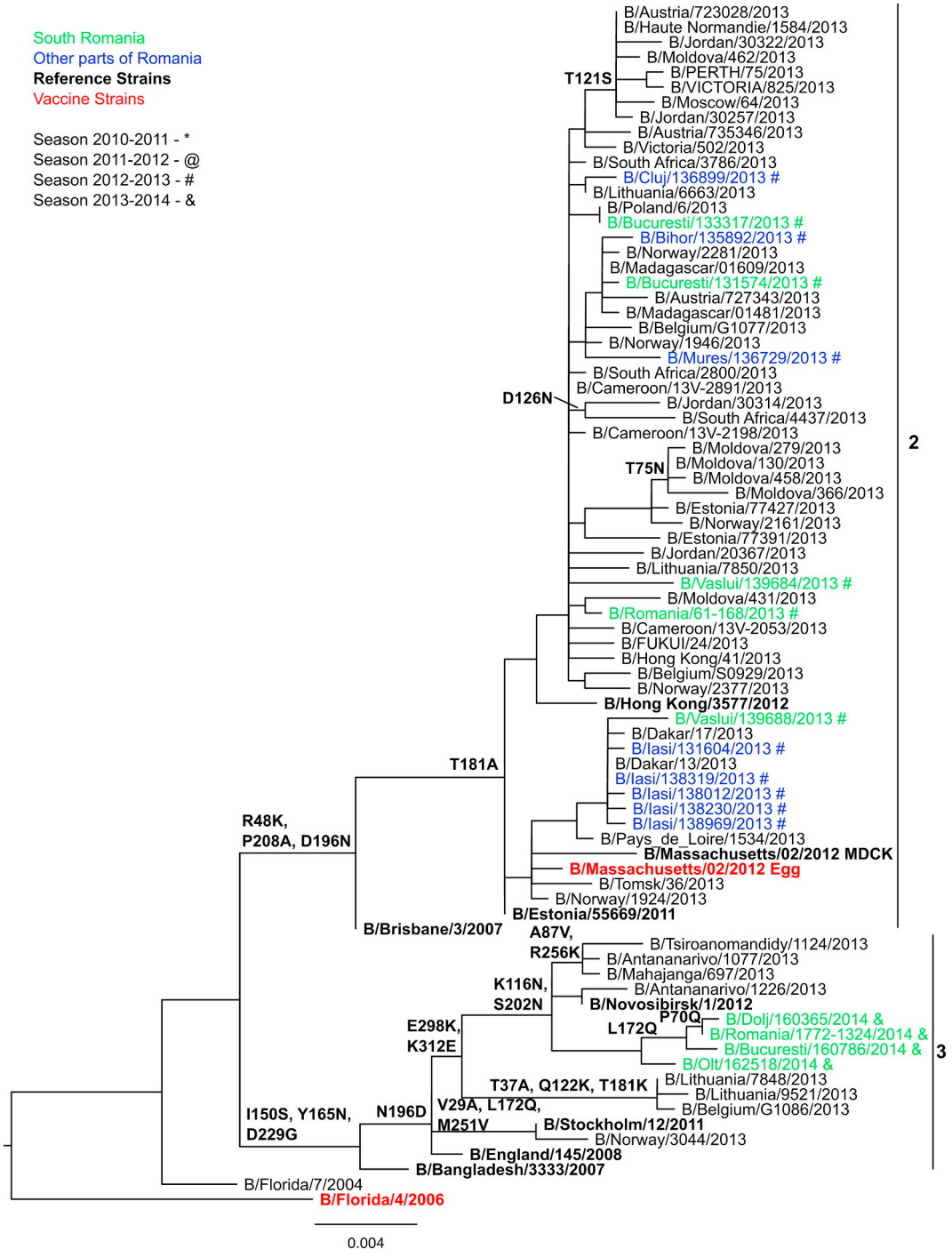

Fig. 5. Phylogenetic tree of influenza type B genes from the South Romania and the rest of the country in relation to sequences from rest of the Europe. GISAID accession numbers: B/Iasi/131604/2013 EPI439279; B/Bucuresti/131574/2013 EPI439281; B/Cluj/136899/2013 EPI464958; B/Iasi/138012/2013 EPI464959; B/Iasi/138230/2013 EPI464960; B/Iasi/138969/2013 EPI464963; B/Vaslui/139684/2013 EPI464964; B/Vaslui/139688/2013 EPI464965; B/Iasi/138319/2013 EPI464968; B/Bihor/135892/2013 EPI464969; B/Mures/136729/2013 EPI464970; B/Romania/61-168/2013 EPI464971; B/Bucuresti/160786/2014 EPI516552; B/Romania/1772-1324/2014 EPI516553; B/Dolj/160365/2014 EPI516555; B/Olt/162518/2014 EPI516556; B/Bucuresti/133317/2013 EPI460588.

(Kissling et al., 2013). WHO recommend changing the A/H3N2 strain in the composition of influenza vaccine for the following year.

The 2012–2013 influenza season was marked by the return of A/(H1N1)pdm09 which hardly circulated in Europe in the previous season, but also by a mixed influenza virus circulation. The 2012–2013 influenza season in Romania was unusually long, spanning from the first week of 2013 up to week 17, but similar to the majority of European countries. A close co-circulation of A(H1N1)pdm09 and influenza type B/Yamagata lineage viruses was recorded during the whole season, and relatively few A(H3N2) detections (Pitigoi et al., 2015). Although the A/H3N2 vaccine effectiveness improved over the previous season, it remained low to moderate at 42.2% despite the replacement of A/H3N2 vaccine strain, while A(H1N1)pdm09 IVE was 50.4% and 49.3% for influenza B (Kissling et al., 2014).

The 2013–2014 influenza season was mild and dominated by influenza A viruses, with a co-circulation of A(H1N1)pdm09 and A(H3N2) viruses in most European countries. In Romania, A/H3N2 was by far the most detected virus, but co-circulated with A/(H1N1)pdm09

during the epidemic, and with a few influenza type B detections from the midseason. This situation was at odds with the majority of European countries, where it was the first season after 2010/11 that had A(H1N1) pdm09 reaching over 50% of subtyped strains (European Centre for Disease Prevention and Control, 2014). The characterized strains revealed limited antigenic and genetic variability that generally translated to a close vaccine strain match.

Due to the geographic position, Romania registered the peak of ILI/SARI detection later than the rest of Europe in the four post-pandemic influenza seasons – explained by the usual influenza west-to -east progression observed during previous pre-pandemic seasons (1999–2007) (Paget et al., 2007). The same pattern of influenza transmission was maintained for A(H1N1)pdm09 in the post-pandemic period up to 2015 (Caini et al., 2017) Antigenic characterization indicated limited antigenic variability of influenza A(H1N1)pdm09 virus despite gradual accumulation of point mutations in HA gene. Mutation D222N within the HA RBS appears to have some association with severe clinical manifestations (Ruggiero et al., 2013) (Vazquez-Perez et al., 2013). Apart from this substitution, no other mutation associated with the severity of infection was noticed during the four influenza seasons analyzed.

5. Conclusions

During the first four 2009 post-pandemic influenza seasons in South Romania, as well as in the rest of the country, we observed a mixed picture of A(H1N1)pdm09 co-circulation with either A/H3N2 or influenza B, the only exception being season 2011–2012 when A/H3N2 clearly dominated over the other viruses. Young and mature adults were the most affected age group, for both ILI and SARI cases, with fatality rates higher in the severe cases. Obesity and heart and blood vessels chronic diseases were the dominant comorbidities in the severe infections with the A(H1N1)pdm09 subtype.

Antigenic and genetic characterization of influenza virus from the first four post-pandemic seasons showed that circulated strains in South Romania and in the rest of the country generally matched those included in the trivalent vaccine. Phenotypic and genotypic analysis of isolates that was used to monitor de presence of antiviral (Oseltamivir and Zanamivir) resistance, revealed that all isolates were susceptible to both drugs.

Influenza circulation profile in South Romania was consistent with the rest of the country and with the rest of Europe, while antigenic and genetic characterization of influenza virus collected in South Romania contributed to a better sampling of circulating viruses that translated in a more accurate picture of variants circulating at national level. The results of this study highlight the importance of influenza virus surveillance should be sustained and reinforced by additional public health programs in South Romania and at national level.

6. Limitation of the study

During the study ILI data from sentinel was likely underreported, and was the reason we had to rely on routine data. Also the low number of GPs participating in the study, only 98 but they covered 15/20 districts.

Disclosure statement

The authors declare no conflict of interest.

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